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Polymer based antibiotics formulation for the treatment of lung infections

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Dedication

To my parents and my dear wife.

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Polymer based antibiotics formulation for the treatment of lung infections

Ju Du, Ph.D.

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Supervisor: Hugh D. C. Smyth

Delivering antibiotics through pulmonary is a promising approach for treatment of cystic fibrosis (CF). For the current marketed antibiotic formulations, however, the requirement of multiple drug administrations per day to achieve a therapeutic effect limits their applicability. To reduce administration frequency, controlled pulmonary release formulation is a strategy which can maintain effective and consistent local drug concentration and therefore prolong the time period between doses. However, these particles of controlled release formulation with optimum aerodynamic diameter range targeted to the alveolar region (i.e. 0.5<da<5 μm) will be rapidly cleared by the alveolar macrophages. This is because the geometric diameters of these particles are usually less than 6 μm, which is the preferable size range for alveolar macrophages' uptake.

To overcome the clearance of alveolar macrophages for the controlled release formulation, the approach we employed in the current study was to form swellable

hydrogel dry powder by utilizing the unique benefits of hydrogel, higher drug payload, larger geometric diameter after swelling, and sustained drug delivery. In the first study, based on the fact that ciprofloxacin could form hydrogel with alginate, a nano-in-micro hydrogel particle formulation was developed for sustained pulmonary drug delivery, which takes the advantages of both chitosan based nanoparticles and swellable and respirable alginate hydrogel particles. The dry nano-in-micro hydrogel particles exhibited a rapid initial swelling within 2 minutes, and showed sustained drug release pattern. When delivered to rats, it enabled ciprofloxacin to achieve a low systemic exposure but maintained higher concentrations in the lung for more than seven hours.

In the second study, we directly combined ciprofloxacin with alginate to form hydrogel dry powder, without the addition of extra chitosan. In such way, we simplified the preparation method for hydrogel particles, decreased the potential risk of polymeric chitosan accumulation in the lung tissue, and increased the ciprofloxacin loading efficiency from 30% to 57% in the finial microsized alginate hydrogel dry powder. Ciprofloxacin was present in the amorphous state in the dry powder and was released in a controlled release manner relative to ciprofloxacin alone, i.e. 80% of drug released at 8 hours.

Despite aggressive antibiotic treatment, the elimination of chronic Pseudomonas aeruginosa (*P. aeruginosa*) infections in CF lungs is extremely difficult. The pathogen often adapts to resist both the host inflammatory defense mechanisms and externally applied antibiotic therapy, often allowing for the formation of microbial biofilms. The resulting biofilms are thick, pathogen embedded, and highly resistant to common therapeutic agents currently used in CF infections. Thus, the development of newer

antimicrobial agents with superior abilities to eliminate the established chronic biofilm associated with CF infections remains the utmost priority in CF therapy. A conventional antibiotic, tobramycin was chemically modified. Tobramycin has previously been demonstrated to bind to biofilm matrices, thus reducing the effective concentration of antimicrobial able to reach the pathogenic organisms, as well as limiting the penetration of the antibacterial agent to the deeper microstructure of the biofilm, thereby creating an undesirable stress response in the pathogen. Modification of antimicrobial as by PEGylation appears to be a promising approach for overcoming the bacterial resistance in the established biofilms of Pseudomonas aeruginosa.

This body of work provides two promising strategies of delivering antibiotics via pulmonary route for the treatment of cystic fibrosis. The first strategy is to form controlled release formulation, typically as swellable hydrogel dry powder, which could sustain the drug release, and swell to larger size as to avoid the alveolar macrophage uptake as to increase the local retention period. The second strategy is related to the biofilm resistance. By modifying the existent antibiotic to reduce the binding efficacy to the extracellular matrix of biofilm, more antibiotic could subsequently enter into the inner region of bacterial colony.

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1.1 ABSTRACT

Inhalation therapy has long been used by humans to treat diseases in the respiratory tract. The primary reason for selecting this method of drug delivery is the ability for the regional targeting of the respiratory tract for local diseases (e.g. asthma, chronic obstructive pulmonary disease, cystic fibrosis). This allows rapid therapeutic onset (i.e. during acute asthma attack), minimal incidence of systemic side effects (e.g. broad side effects observed with long term use of corticosteroids), and achievement of much higher concentrations at the respiratory region (e.g. lung infections). Additionally, the lung contains much lower concentrations of metabolizing enzymes than other portals of entry, especially the gastrointestinal tract, which decreases the likelihood of the degradation of drug. Combining a high surface area (70 – 100 m^2), good permeability through the thin epithelial cell layer and the small fluid volume on the absorption surface in the peripheral respiratory region, small molecules can be rapidly absorbed into the blood stream. These characteristics have led to a number of very successful therapeutic products and treatments that will be introduced below.

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These attributes, however, have also lead to the administration of other compounds via the lung typically via smoke inhalation. Nicotine administration, for example, is an excellent example of an early form of drug delivery (though non-therapeutic) that allows the user to accurately titrate pharmacokinetics. Smoking of course is where pulmonary drug delivery began.

1.2 HISTORY AND RATIONALE OF PULMONARY DRUG DELIVERY

The origins of pulmonary drug delivery may be traced back to at least 4000 years ago in Ayurvedic medicine. During this time in India, many respiratory illnesses were treated by smoking a pipe smeared with a paste made out of the *Datura* species and other herbs. Ancient Egyptians also used to place black henbane, plant of the *Hyosycamus muticus* species containing the anticholinergic compound, hysocyamine, on hot bricks and inhale its vapor (1).

In 1778, John Mudge, an English physician coined the word “inhaler” and published a design of a remedial inhaler (1). His design was widely accepted and used in the ceramic inhalers popularized in the 19th century.

Modern inhalers were introduced with the pressured metered dose inhalers (pMDIs) that were first developed in the 1950s as an alternative to early nebulizers. A pMDI is a device that delivers medication to the respiratory system, in the form of an aerosol spray generated by forcing a liquid through a nozzle under pressure. It consists of a drug dissolved or suspended in a propellant, a liquefied compressed gas. The first pMDI was developed in Riker Laboratories, Inc (now 3M Pharmaceuticals, St. Paul, Minnesota) in 1955. The development was initiated by Dr. George Maison, president of Riker Labs, who licensed a patent on a metering valve invented by Mr. Meshburg. While the

Meshburg valve was initially intended for perfume aerosols, Dr. Maison's asthmatic daughter suggested the potential use for it in pharmaceutical inhalation therapy. More recently, dry powder inhalers (DPIs) have become commonplace. These devices have emerged from the environmental concerns associated with the pMDIs. The transition from the chlorofluorocarbons (CFCs) to hydrofluoroalkane (HFCs) was not straight forward and therefore several large pharma companies opted to have DPI development capabilities. These devices are generally patient activated rather than device activated and therefore do not suffer from issues of inhalation coordination with actuation of the aerosol. They also have the ability to have improved stability, increased administration dose, and have shown to have marketing benefits. Advair™ for example, is a combination drug DPI that has been a commercial success, $4.7 billion in 2010.

1.3 CURRENT PRODUCTS AND DISEASES

1.3.1 Asthma

Asthma is an inflammatory disease associated with reversible narrowing of the bronchial airways (2). The exact causes of asthma have not yet been determined but are likely linked to the environment, genetics and biology. Asthma affects more than 22 million people in the United States and 300 million people worldwide. It results in the deaths of approximately 255,000 people globally each year.

The treatment of asthma can be broadly divided into two categories based on the drug's effect. Firstly, bronchodilators induce relaxation of the airway smooth muscle. Secondly, anti-inflammatory agents are used to treat underlying airway inflammation. In practical terms, however, clinicians prefer to classify these medicines with respect to their treatment onset time, i.e. relievers (acute use and effects) and preventers/controllers

(chronic use and effects) respectively.

Short-Acting β-agonists, including albuterol, levalbuterol, and pirbuterol are available in several different commercial formulations, such as the metered-dose inhaler (Ventolin® HFA, ProAir® HFA, Proventil® HFA), or solution for nebulization (AccuNeb®). Long-acting β agonists (LABAs) act in the similar mechanism as short-acting β agonists, which can reduce the inflammation and open the airway. However, the LABAs have long duration of activity, up to 12 hours (2, 3). LABAs include salmeterol (Serevent® Diskus®) in a multidose dry powder inhaler, formoterol (Foradil® Aerolizer®) in a single dose dry powder inhaler. Long-term control medicines are used to reduce the chronic inflammation in airways. Types of long-term control drugs include: inhaled corticosteroids, leukotriene modifiers, long-acting beta agonists (LABAs), and theophylline. Inhaled corticosteroids have shown the greatest effect in controlling asthma symptoms, because they exhibit multiplicity of anti-inflammatory activities via the transcription of genes (4, 5). As with most asthma therapeutics, there are different formulations available for corticosteroids. Firstly, in the form of HFC metered-dose inhalers, there are beclomethasone (e.g. QVAR®), ciclesonide (Alvesco®), fluticasone, budisonide with formoterol (Symbicort®), and Fluticasone and Salmeterol (Advair®). Secondly, in the form of dry powder inhalers including budesonide (Pulmicort Flexhaler®), mometasone (Asmanex® Twisthaler®), Fluticasone and Salmeterol (Advair Diskus®). More detailed information on marketed products, information in the following reference provides a current review (2, 5).

1.3.2 Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is referred as a group of lung

diseases, including chronic bronchitis and emphysema. Most patients with COPD have both conditions at the same time. Commonly there are three causes of COPD, including smoking, inhaled toxins or other irritants, and genetic predisposition. The damage caused in lungs cannot be reversed in COPD, therefore controlling symptoms and minimizing further damage is the main objective of the treatment (6); The most important approach for patients is smoking cessation (7). Medicines used to treat COPD include bronchodilators, inhaled steroids, antibiotics and vaccines.

Bronchodilators can relax the smooth muscles around airways, making breathing easier. These medicines commonly used in treating COPD were listed in reference (8). Other treatments include antibiotics, anti-inflammatory, vaccines, mucolytic agents, antioxidant agents, etc.

1.3.3 Cystic fibrosis

Cystic fibrosis is a genetic disease caused by the mutation of the cystic fibrosis transmembrane regulator gene (CFTR), leading to the abnormal movement of ion and water in the airway epithelial. Consequently, the patients with cystic fibrosis will experience the accumulation of mucus, bacterial infection, inflammation, and even tissue destruction (9). Currently, there is not a specific cure for cystic fibrosis.

Pulmonary infections are the common disease manifestation observed in patients with cystic fibrosis. Antibiotics, either taken by oral or, more commonly, via inhalation are typical treatments. Nebulized tobramycin and colistin are the choices for the treatment of infection caused by the *P*.aeruginosa. A significant problem is the deposition of those antibiotics, since most of them were found in the conductive zone instead of respiratory zone (10). Tobramycin and colistin in dry powder inhaler formulations offered

a new approach to the treatment of infection (10-12). Aztreonam lysine (13-15), which is a monobactam antibiotic for Gram-negative organisms, is taken in the form of nebulization, and is under phase III study (10).

In addition to treatment of infections, CF patients may receive muco-active agents. Thick mucus is generally observed in the lungs, and its accumulation facilitates infection and inflammation. Currently, two choices are available to control airway mucus aiming to thin the mucus to allow for easier clearance. Acetadote®, which contains the acetylcysteine as the active ingredient, disrupts the intermolecular bonds of the mucus polymer, thus lowering the viscosity and elasticity of the mucus. Dornase Alfa, commercially named as Pulmozyme®, is administered via nebulization and can reduce the viscosity of cystic fibrosis sputum in a dose dependent manner by cleaving the DNA present in the airway mucus (16-18). Other candidates are the gelsolin and thymosin, which are both in development. Mannitol has been used to improve hydration of airway mucus via a hyper-osmotic effect, resulting in the decreasing of the viscosity of airway mucus. This product, along with nebulized hypertonic saline in now approved for use in many countries.

Due to the genetic cause of cystic fibrosis, many researchers have therefore focused on gene therapy. Development of a successful gene therapy has been problematic. A complex composed of DNA and cationic lipids, which was delivered through aerosolization or direct instillation, showed a limited improvement in the treatment of cystic fibrosis (9, 19). Virus vectors, yielding a high transfection rates, have been limited because of the immunogenic issues (20-22). Despite the lack of practical success in the clinic, gene therapy remains a promising approach for the treatment of

cystic fibrosis.

1.4 RATIONAL FOR DEVELOPING POLYMER BASED PULMONARY DELIVERY SYSTEMS

Few polymers are currently used in commercially available aerosol formulations. In particular, the pulmonary route is characterized by the few excipients that are found in approved products. However, despite the lack of commercialization of polymeric systems for lung delivery there has grown a large body of research that has focused on polymeric drug delivery systems for airway administration.

1.4.1 Drug Protection

Almost all of the metabolizing enzymes that exist in the liver are also found in the lungs, but often at lower levels. In addition, the metabolizing activity of those enzymes in the lungs is lower than the liver (23). However, many inhaled drugs are regarded as substrates of enzymes existed in lungs, for example: budesonide, salmeterol (24), and ciclesonide (23, 25, 26).

Owing to these issues of local metabolism, polymers may be employed to protect drugs from enzymatic degradation, increasing local or even systemic bioavailability where appropriate. For example, poly (l-lactic acid) (PLA) coated budesonide, delivered through the intratracheal instillation to rats, showed a sustained release profile and a higher pulmonary-targeted effects (27). Additionally, another group used PEG(5000)-DSPE polymeric micelles containing budesonide, which was compared to Pulmicort Respules®. And the in vivo study indicated a longer period of inhibition toward inflammatory cells in the asthmatic rats (28).

It is well known that insulin is sensitive to enzymatic degradation, and several researchers have attempted to develop inhaled insulin systems. For instance, one group developed an insulin loaded polybutylcyanoacrylate nanoparticle system which was also delivered via the intratracheal route. Compared with insulin solution, these polymeric particles significantly prolonged the pharmacodynamic action of insulin toward plasma glucose levels (29).

In gene delivery, prevention of degradation of the gene prior to its targeting specific cells is an important objective. Polymeric formulations have therefore been applied in gene delivery. For example, researchers developed an acid degradable cationic polymer, which enabled condensation of anionic DNA. In contrast to naked DNA, these degradable polymers could achieve a significantly enhanced gene expression (30).

Similar with DNA, siRNA is degraded quickly in biological environments such as the plasma and cellular cytoplasm (31). Ensuring siRNA stability during transit to the target site is one the major challenges that remains to be overcome for successful delivery of these molecules. As such, appropriate formulation systems are needed for stabilizing siRNA, enhancing the retention time in the lung region, and improving therapeutic effect. With this aim, polymers have also been applied for pulmonary siRNA delivery. Chitosan, which is well explored for drug delivery systems, has been regarded as a good candidate for gene delivery, because of its protection on siRNA, and improvement on genetic transfection (32-35). Other polymers used widely in the siRNA delivery included PLGA (36-38).

Besides enzymatic degradation, another two main factors influencing the fate of drugs are mucociliary clearance and alveolar macrophage uptake. To avoid the

mucociliary clearance, the commonly adopted method is to utilize the mucoadhesive polymers such as chitosan (39-41) and PLGA (42) or to avoid the muco-ciliary escalator by aerodynamically targeting the deeper regions of the lungs. Mucoadhesive polymers would adhere to the mucus for a longer period, and hence increase the retention time in the respiratory system. In addition, PEG seems to prolong the residence time of the drugs in lungs by reducing their degradation and engulfment by alveolar macrophages (43). Conjugation to 5 kDa poly(ethylene glycol) (44), has also been shown to facilitate particle penetration of human mucus, potentially decreasing the effect of mucociliary clearance.

In the case of avoiding alveolar macrophage clearance, one way is to form the larger porous particle with polymer (45-48), since larger porous particle exhibited a suitable aerodynamic diameter which allows the particle reach into the deep lung while its geometric size is not optimal for macrophage to uptake. Another way is to get aid of endogenous agents, such as hyaluronic acid (HA). A study, in which an inhaled microparticle system was formulated through co-spray drying of insulin and HA, showed that this system displayed a longer mean residence time (MRT) and terminal half-life ($t_{1/2}$) compared to spray dried pure insulin (49).

1.4.2 Drug release and targeting

Application of polymeric particles in drug delivery through pulmonary routes has been reasonably well studied due to the several advantages that is offered by this approach including for example sustained release, reduced dosing frequency, proper aerodynamic size and good bioavailability.

Polymers used for aerosol formulations can be divided into two categories based

on their origin. The first type is natural polymers, for example: albumin, carrageenan, chitosan, gelatin, and hyaluronic acid. The other type are synthetic polymers, including for example poly (lactic acid), oligo (lactic acid), poly (vinyl alcohol), and acrylic acid derivatives (50).

Additionally, polymers are usually deployed as particulate systems as either nanopartilces or microparticles. Compared with oral administration or injection, nanoparticle delivery to the lungs is less advanced from a development stage piont of view. The primary depostion mechanism of nanoparticle sized aerosols in the respiratory tract is via Brownian motion. Due to this longer times are needed to increase the depostion efficiencies and to avoid exhalation of the nano-aerosols (51, 52). In addition, generation of sufficient numbers of nanoparticles that carry a sufficient payload of drug is quite challenging. However, due to the wide and tunable properties of many polymers there is intensive research efforts now directed toward using the polymeric nanoparticles in the lung. Nanoparticles administered to the airways are often incorporated into the microparticles. Through specific manufacture methods such as spray-drying, the combined formulation facilitates appropriate aerodynamic diameters for lung delivery while taking advantage of some of the potential advantages of nanoparticles (53, 54). These advantages may include size dependent phenomena. For example, nanoparticles with a size lower than 150nm, experience delayed lung clearance, and may facilitate increased drug absorption compared with larger particles (55, 56). Several polymeric nanoparticle systems for pulmonary drug delivery have been recently reviewed (51).

In contrast to polymeric nanoparticles, polymeric microparticles have seen broader applications thus far primarily due to their higher drug loading capacity. Many

studies have shown that microparticles were good carrier candidates for pulmonary drug delivery (57-59).

Polymeric systems have been shown to improve the duration of effect of inhaled drugs either for local or systemic therapy (46, 60, 61). Generally, the sustained or controlled release of active agents from polymeric carriers depends on their distribution within the particles and the degradation rate of the polymer. Clearly the release mechanims of polymeric delivery systems depolyed in the pulmonary route will be similar to those used in classical controlled release drug delivery applications. Specifically three release mechanisms may be reponsible for drug release from these systems, including drug diffusion, polymer swelling followed by diffusion and polymer degradation (62) .

In the situation of drug diffusion, the polymer systems are stable and have a general absence of swelling and degradation, such that the drug molecules pass through the polymeric matix to be released (62-64). In the case of swelling polymers, the polymers take up fluid from the environment, leading to matrix swelling. The increased pore size in the matrix subsequently facilitates drug transportation from the delivery system. When the polymers degraded in the medium due to hydrolysis or bulk erosion, the release of the drug is accompanied with the decreasing of molecular weight of polymers (62).

1.4.3 Vaccines (41)

Respiratory tract is a main route for many pathogens to enter into the human body,

causing short or chronic diseases (65-69). Because of its large surface area in the alveolar region and thin epithelial surfarce that is heavily monitored by the immune system, it is a promising region to target vaccines. A large number of alveolar macrophages and dendritic cells exist in the deep lung and may result in excellent immune responses. Even so, a question remains in pulmonary vaccination as to the deposition site of antigen in the respiratory tract. Many vaccines delivered via intranasal route have been developed (70, 71). Intranasal vaccination is dependent on targeting the relatively smaller surface area in this region and the complexity of the nasal geometry The main local immune response to intranasal vaccines originates from the nasal associated lymphoid tissue (NALT) (72, 73). In addition, it is well known that respiratory tract offers a large surface area for aerosol targeting, including for vaccination. Alveolar space may be an ideal target for vaccination; this is due to its high permeability to macromolecules, as well as the adequate number of immunological cells. For example, delivery of (74) a polysaccharide vaccine into the alveoli region triggered a higher level of IgG antibody response than was observed in bronchial vaccination which targeted the upper airways. This maybe by virtue of the large surface area differences between the alveoli and bronchial regions, where the former is around 100 m^2, while the latter is 1 m^2. However, a later study failed to demonstrate the enhanced pulmonary response, leading to a further study of polysaccharide vaccine in the upper and lower airway (75, 76). Another research effort focusing on influenza vaccines and regional deposition differences in the lung indicated that deep lung immunization could achieve higher antibody level, in both local and serum (77). A recent study highlighted the function of M cell role in the immune response in the upper respiratory tract. It was suggested that respiratory M cells were essential to trigger

the systemic and local immune responses (78).

In the views of pulmonary vaccine development, we usually consider the following points: targeting specific cellular organs, enhancing delivery and achieving higher expression levels of antibody, and finding effective vaccine adjuvant with a promoting immune response. And a growing number of synthetic and natural polymers have been used as an adjuvant in pulmonary vaccine, since they can promote the uptake of antigens by antigen-presenting cells (APCs) (79). In the category of synthesis polymers, PLA (79, 80)and PGLA (81-83) are well studied examples. A recent study (84), which utilized the PLA and PLGA as adjuvants in the form nanoparticles, investigated the influence of surface charge, particle size, and surface hydrophobicity, on immune responses. And the results revealed that alveolar macrophages seemed to prefer phagocytosis of larger hydrophobic particles instead of smaller particles.

In the category of natural polymers, chitosan has been well studied. It has been developed into many formulations for vaccination, including chitosan-based polymers, chitosan-based micro and nanoparticles, chitosan based formulations containing adjuvants, and chitosan-coated particles. For example, a recent report of a modified chitosan, N-Trimethyl chitosan (TMC), microparticles vaccine containing diphtheria toxoid was studied following pulmonary administration and showed detectable IgA and IgG levels (85). However, another study indicated an opposite response when using chitosan (86). When applied as adjuvant in adenovirus vaccine, chitosan reduced the immune responses in vivo via the negative impact on the $CD8^+$ T cells. Thus, more research is needed for a clear mechanisms for chitosan specifically, and polymers in general, for pulmonary vaccination.

1.5 PHYSIOLOGICAL BARRIERS TO LUNG DELIVERY

1.5.1 Aerodynamics

There are still several physiological barriers that need to be considered when delivered the drug through the pulmonary route. The aerodynamic diameter of aerosol particles significantly influences the drug deposition and retention in different lung regions (87-89). The aerodynamic diameter is related to the geometric diameter and particle density (90, 91).

The differences in aerodynamic size of particle results in differential deposition along various locations in the respiratory region. For example, particles with aerodynamic diameters bigger than 10 μm, will mostly deposit in the upper airway, including mouth, throat, and larynx. Particles with aerodynamic diameters lower than 0.5μm, are often exhaled due to the short time given for deposition via diffusion of the particles in the alveoli region. Particles with aerodynamic diameters of 1-5μm can deposit in the alveolar region and are generally the target size for lung delivery for the diseases mentioned above (92, 93).

Most research has focused on particles within size range of the 1-5μm for lung delivery, which is also referred as the "respirable" size range. One research trend over the past decade or so has been to develop large porous particles, that have a low density but large geometric size (45, 48, 59, 94). For instance, via a double-emulsion method, PLGA microparticles were made with ammonium bicarbonate that is converted into ammonia and carbon dioxide gas, thus resulting in a highly-porous particle during particle formation. This type of porous particle could be applied for encapsulating both low molecular weight and macro molecules, such as doxorubicin·HCl and lysozyme. In the

case of doxorubicin, a sustained release profile was observed and 52% of drug was released over 4 days (59). Another example (48) showed the application of porous particle in an animal model. The author encapsulated the complex of prostaglandin E1 (PGE1) and 2-hydroxypropyl-β- cyclodextrin (HP-β-CD) into a porous PLGA microparticle system. In this formulation, prostaglandin E1 (PGE1) was used to treat hypertension, while HPβCD, was selected as an osmotic agent to generate the pores in the particles surface. The in vivo results indicated that, this porous particle with a aerodynamic diameters of 1 to 5μm, exerted a prolonged release of PGE1 after intratracheal administration.

1.5.2 Mucociliary clearance

When deposited in the respiratory tract, drugs and drug carrying particles need to overcome efficient biological barriers before reaching the target site, whether is the epithelia or the blood circulation.

Mucus barrier, which form part of the system that results in mucociliary clearance, is the first obstacle in the pulmonary delivery. Mucus layer mostly covers the airway epithelium which contains ciliated cells, secretory cells and basal cells. The mucus layer covering the conducing airways is about 5-10 μm thick (56, 95), and is mainly composed of mucins and glycoproteins (96). These two components contribute significantly to the binding ability of the mucus layer, in terms of electrostatic, hydrophobic, and hydrogen bonding interactions, which are all responsible for the trapping of drug or particles. There is a competition between the mucociliary clearance and dissolution followed by absorption. Dissolved material may cross the mucus layer by diffusion and reach the epithelial cell layer and thus avoid of mucociliary clearance.

While, the particles with slow dissolution or poor drug release (97, 98), are likely to be cleared via mucociliary clearance.

The mucus layer functions as a physical, biochemical and immunological barrier. It may prevent the drug from reaching the epithelia, limiting its therapeutic effects. Therefore, avoidance of the mucociliary clearance attracted much attention among researchers. The first way is to disrupt the mucus layer with particles which could open new diffusion pathways via the interaction between the mucus and particle (44). Researchers (96) showed polystyrene particles and diesel particulate matter caused the disruption of mucus layer allowing increased drug permeation.

Since mucus is adhesive, several researchers are developing mucoadhesive particles for lung delivery (99). It has been proposed that by utilizing the interaction between the mucus and particle, prolonged retention time of particles, may allow more drug diffusion through the mucus leading an increasing absorption and bioavailability (9).

Polymers with mucoadhesive properties such as chitosan have been investigated. Chitosan exhibits bioadhesive properties that promote permeation, absorption of drug, as well as being a relatively biocompatible polymer (41, 100-105).

1.5.3 Alveolar macrophages

The clearance of particles which have reached the deep lung, specifically the lower alveolar region, occurs via the alveolar macrophages. Alveolar macrophages are abundant in the deep lung region, and more than 90% of these cells are located around

the alveolar septal junctional zones (106-108). On the surface of alveolar macrophages, many receptors exist, such as the immunoglobulin receptor (fcR), complement receptor (CR), mannose receptor (MR) and several types of scavenger receptors (106). In the cases of macromolecules, alveolar macrophages hinder the absorption to the lung circulation (109), because macromolecules are absorbed slowly and the prolonged transport process enables the alveolar macrophages to phagocytose the drug, thus reducing the bioavailability. Usually, the prolonged transportation happen in the molecules with molecular weight above 40 kDa (110). It seems that proteins with a lower molecular weight, approximately less than 25 kDa, were less influenced by alveolar macrophages (110, 111). For example, insulin (MW=5807) did not show a increase absorption even when the alveolar macrophages were disrupted (109, 111).

Generally, alveolar macrophages efficiently engulf particles in the range 0.5-5 μm geometric size, which more or less overlaps with the respirable particle size range when particle density is one. Various strategies have been published for overcoming the uptake by alveolar macrophages. One approach is to form nanoparticles with geometric size less than 0.1μm (112, 113). Results showed that nanoparticles could escape the uptake by alveolar macrophages. In contrast to decreasing the particle size, another way is the development of large particles, which exhibit the large geometric size (more than 5 μm), but have low density, therefore performing like aerodynamically smaller respirable particles (45). The large geometric size of porous particles could reduce the clearance by alveolar macrophages (114, 115).

Similarly swellable microparticles achieve aerodynamic properties allowing deep lung delivery but geometric properties that minimize macrophage uptake (54). This

formulation has respirable aerodynamic sizes when dry but large geometric sizes when swollen after being exposed to the moist lung epithelia lining fluid. Such characteristic enables this formulation evade macrophage uptake and show a sustained release profile through a controlled polymeric architecture.

1.5.4 Drug absorption rates

Generally, there are two types of epithelial cells in the respiratory system, and they are airway and alveolar epithelium (41), respectively. The airway epithelium mainly located at the upper/central respiratory tract. For the drug deposited in these regions, before being absorbed into the circulation of lung, they have to overcome two barriers, which are the thick mucus layer covering on the surface and the tight junctions between the epithelial cells. And the airway epithelial cells form a layer which is about 80 μm in the trachea region and decreases to around 10 μm in the region of bronchioles (110, 116). Besides the airway epithelium, micro-fold cells (M-cells) are found. M-cells are response for the uptake and transport of antigens within the mucosa-associated lymphoid tissue, leading to the immune activity in the upper respiratory tract (41, 117-119). In a recent study, the author delivered an antigen intranasally to mice, and found that M cells could take up the antigen and induct the immune responses, which was nasopharynx associated lymphoid tissue independent process (78).

Alveolar epithelial cells are found in the distal respiratory tract. In the alveolar region, the mucus layer is replaced by the pulmonary surfactant, its thickness is around 0.07 μm (110, 116). And the alveolar epithelial cells are composed of type I and II pneumocytes. Type I cells covers about 95% of the alveolar surface (120, 121), and form a thin layer of 0.05 μm thickness. And there is gap of around 1 nm between type I cells

(122). Type II cells are mainly in responsible for the release of surfactant. Compared with the 0.25 m^2 area formed by the airway epithelium cells, the surface area in the alveolar region is about 70-100 m^2 (121, 123, 124).

In the alveolar region, epithelial cells form a much tighter barrier toward the absorption of the compounds than the pulmonary capillary endothelium (116, 122). For hydrophilic agents with lower molecular weight, they usually can be absorbed in the bloodstream within minutes (125-127), and are considered to be transported through transcellular diffusion (107, 116). However, if molecules that are insoluble due to the high hydrophobicity, it may take weeks for them to be absorbed (128).

Hydrophilic molecules may be absorbed either via the transporters or through the tight junctions (107, 116). If the hydrophilic molecules exhibit neutral or negative charge and a small molecular weight, generally, they will be absorbed quickly within 60 min (107, 128).

Even though it is still not clear which route is response for insulin absorption, the popular idea is via paracellular diffusion (107, 116). In addition, scientists suggest that the absorption of insulin or small peptides occurs at the distal airways just before the alveoli, since, with respect to the electrical resistance, the tight junctions at the distal airway are the lower than that of the trachea and alveolar region (107, 129). Furthermore, it appears that the number of tight junctions in upper airways are as much as five times higher than that in alveolar region (107, 116). Therefore, the ideal place for absorption of small peptides is the deep lungs (107, 125).

The bottleneck for the absorption of macromolecules is the size (107). In a study investigating the permeability of dextran across the alveolar epithelium, the author

assessed the transport of dextran with different molecular weights (130). There was an inverse relationship between the permeability coefficient and molecular weight as expected. If the molecular weight of dextran was within the range of 4-40 kDa, in which the molecule had a radius of less than 5 nm, it seems that dextrans were transported through the epithelium via paracellular diffusion. While, the dextrans with the molecular weight between 70 kDa and 150 kDa, having a radius greater than 6nm, likely adopted other pathways to cross the epithelia, such as pinocytosis.

In addition, receptor-mediated transcytosis or others, may be responsible for the transport of macromolecules and do not have apparent size-dependent transportation across the epithelium (121, 131). For example, there is still controversy in the transport of albumin. One accepted point is that the absorption of albumin was mediated through a specific binding protein expressed in the alveoli region (121, 132, 133).

1.6 SUMMARY

The lung is an excellent organ for the administration of a number of therapeutic agents due to the unique of method access to the lung tissue and the underlying rich blood supply. However, as evidenced by the discussion above, the lung poses significant challenges to drug delivery both anatomically and physiologically. Although a number of successful products have been developed and have had great impact on health care of millions, the next generation of inhaled therapeutics will have to achieve improved navigation through the complex cellular and molecular barriers recently elucidated within the lung. In the following chapters we will discuss how polymeric delivery systems are leading the technology advancements in this pursuit.

1.7 REFERENCES

1. Anderson PJ. History of aerosol therapy: liquid nebulization to MDIs to DPIs. Respiratory care. 2005;50(9):1139-50. Epub 2005/08/27.

2. Fanta CH. Asthma. N Engl J Med. 2009;360(10):1002-14. Epub 2009/03/07.

3. Pearlman DS, Chervinsky P, LaForce C, Seltzer JM, Southern DL, Kemp JP, et al. A comparison of salmeterol with albuterol in the treatment of mild-to-moderate asthma. N Engl J Med. 1992;327(20):1420-5. Epub 1992/11/12.

4. Barnes PJ. How corticosteroids control inflammation: Quintiles Prize Lecture 2005. Br J Pharmacol. 2006;148(3):245-54. Epub 2006/04/11.

5. van der Velden VH. Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma. Mediators Inflamm. 1998;7(4):229-37. Epub 1998/10/29.

6. Chronic obstructive pulmonary disease. http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001153/: U.S. National Library of Medicine; 2012.

7. NIH. How Is COPD Treated? http://www.nhlbi.nih.gov/health/health-topics/topics/copd/treatment.html: U.S. Department of Health & Human Services; 2012.

8. Global Strategy for Diagnosis, Management, and Prevention of COPD (Revised 2011). http://www.goldcopd.org/: 2011 Global Intiative for Chronic Obstructive Lung Disease, Inc.; 2011.

9. Roy I, Vij N. Nanodelivery in airway diseases: challenges and therapeutic applications. Nanomedicine : nanotechnology, biology, and medicine. 2010;6(2):237-44.

Epub 2009/07/21.

10. Hoiby N. Recent advances in the treatment of Pseudomonas aeruginosa infections in cystic fibrosis. BMC Med. 2011;9:32. Epub 2011/04/06.

11. Geller DE, Konstan MW, Smith J, Noonberg SB, Conrad C. Novel tobramycin inhalation powder in cystic fibrosis subjects: pharmacokinetics and safety. Pediatr Pulmonol. 2007;42(4):307-13. Epub 2007/03/14.

12. Westerman EM, De Boer AH, Le Brun PP, Touw DJ, Roldaan AC, Frijlink HW, et al. Dry powder inhalation of colistin in cystic fibrosis patients: a single dose pilot study. J Cyst Fibros. 2007;6(4):284-92. Epub 2006/12/23.

13. McCoy KS, Quittner AL, Oermann CM, Gibson RL, Retsch-Bogart GZ, Montgomery AB. Inhaled aztreonam lysine for chronic airway Pseudomonas aeruginosa in cystic fibrosis. Am J Respir Crit Care Med. 2008;178(9):921-8. Epub 2008/07/29.

14. Kirkby S, Novak K, McCoy K. Aztreonam (for inhalation solution) for the treatment of chronic lung infections in patients with cystic fibrosis: an evidence-based review. Core Evid. 2011;6:59-66. Epub 2011/10/25.

15. Daddario MK, Hagerman JK, Klepser ME. Clinical perspective on aztreonam lysine for inhalation in patients with cystic fibrosis. Infect Drug Resist. 2010;3:123-32. Epub 2010/01/01.

16. Henke MO, Ratjen F. Mucolytics in cystic fibrosis. Paediatr Respir Rev. 2007;8(1):24-9. Epub 2007/04/11.

17. Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. Proc Natl Acad Sci U S A.

1990;87(23):9188-92. Epub 1990/12/01.

18. Amin R, Ratjen F. Cystic fibrosis: a review of pulmonary and nutritional therapies. Adv Pediatr. 2008;55:99-121. Epub 2008/12/04.

19. Zabner J, Cheng SH, Meeker D, Launspach J, Balfour R, Perricone MA, et al. Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia in vivo. J Clin Invest. 1997;100(6):1529-37. Epub 1997/09/18.

20. Ratjen F. New pulmonary therapies for cystic fibrosis. Curr Opin Pulm Med. 2007;13(6):541-6. Epub 2007/09/29.

21. Tosi MF, van Heeckeren A, Ferkol TW, Askew D, Harding CV, Kaplan JM. Effect of Pseudomonas-induced chronic lung inflammation on specific cytotoxic T-cell responses to adenoviral vectors in mice. Gene Ther. 2004;11(19):1427-33. Epub 2004/08/06.

22. Moss RB, Milla C, Colombo J, Accurso F, Zeitlin PL, Clancy JP, et al. Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial. Hum Gene Ther. 2007;18(8):726-32. Epub 2007/08/10.

23. Olsson B, Bondesson E, Borgstrom L, Edsbacker S, Eirefelt S, Ekelund K, et al. Controlled Pulmonary Drug Delivery. New York: Springer Science+Business Media; 2011.

24. Cazzola M, Testi R, Matera MG. Clinical pharmacokinetics of salmeterol. Clin Pharmacokinet. 2002;41(1):19-30. Epub 2002/02/05.

25. Nave R, Fisher R, Zech K. In Vitro metabolism of ciclesonide in human lung and liver precision-cut tissue slices. Biopharm Drug Dispos. 2006;27(4):197-207. Epub

2006/03/28.

26. Tunek A, Sjodin K, Hallstrom G. Reversible formation of fatty acid esters of budesonide, an antiasthma glucocorticoid, in human lung and liver microsomes. Drug Metab Dispos. 1997;25(11):1311-7. Epub 1997/11/14.

27. Arya V, Coowanitwong I, Brugos B, Kim WS, Singh R, Hochhaus G. Pulmonary targeting of sustained release formulation of budesonide in neonatal rats. J Drug Target. 2006;14(10):680-6. Epub 2006/12/13.

28. Sahib MN, Darwis Y, Peh KK, Abdulameer SA, Tan YT. Rehydrated sterically stabilized phospholipid nanomicelles of budesonide for nebulization: physicochemical characterization and in vitro, in vivo evaluations. Int J Nanomedicine. 2011;6:2351-66. Epub 2011/11/11.

29. Zhang Q, Shen Z, Nagai T. Prolonged hypoglycemic effect of insulin-loaded polybutylcyanoacrylate nanoparticles after pulmonary administration to normal rats. Int J Pharm. 2001;218(1-2):75-80. Epub 2001/05/05.

30. Ko IK, Ziady A, Lu S, Kwon YJ. Acid-degradable cationic methacrylamide polymerized in the presence of plasmid DNA as tunable non-viral gene carrier. Biomaterials. 2008;29(28):3872-81. Epub 2008/07/01.

31. Sioud M. On the delivery of small interfering RNAs into mammalian cells. Expert Opin Drug Deliv. 2005;2(4):639-51. Epub 2005/11/22.

32. Howard KA, Rahbek UL, Liu X, Damgaard CK, Glud SZ, Andersen MO, et al. RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system. Mol Ther. 2006;14(4):476-84. Epub 2006/07/11.

33. Kong X, Zhang W, Lockey RF, Auais A, Piedimonte G, Mohapatra SS. Respiratory syncytial virus infection in Fischer 344 rats is attenuated by short interfering RNA against the RSV-NS1 gene. Genet Vaccines Ther. 2007;5:4. Epub 2007/02/03.

34. Zhang W, Yang H, Kong X, Mohapatra S, San Juan-Vergara H, Hellermann G, et al. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nat Med. 2005;11(1):56-62. Epub 2004/12/28.

35. Nielsen EJ, Nielsen JM, Becker D, Karlas A, Prakash H, Glud SZ, et al. Pulmonary gene silencing in transgenic EGFP mice using aerosolised chitosan/siRNA nanoparticles. Pharm Res. 2010;27(12):2520-7. Epub 2010/09/09.

36. Bivas-Benita M, Romeijn S, Junginger HE, Borchard G. PLGA-PEI nanoparticles for gene delivery to pulmonary epithelium. Eur J Pharm Biopharm. 2004;58(1):1-6. Epub 2004/06/23.

37. Jensen DK, Jensen LB, Koocheki S, Bengtson L, Cun D, Nielsen HM, et al. Design of an inhalable dry powder formulation of DOTAP-modified PLGA nanoparticles loaded with siRNA. J Control Release. 2012;157(1):141-8. Epub 2011/08/26.

38. Nguyen J, Steele TW, Merkel O, Reul R, Kissel T. Fast degrading polyesters as siRNA nano-carriers for pulmonary gene therapy. J Control Release. 2008;132(3):243-51. Epub 2008/07/16.

39. Yamamoto H, Kuno Y, Sugimoto S, Takeuchi H, Kawashima Y. Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. J Control Release. 2005;102(2):373-81. Epub 2005/01/18.

40. Takeuchi H, Yamamoto H, Kawashima Y. Mucoadhesive nanoparticulate systems for peptide drug delivery. Adv Drug Deliv Rev. 2001;47(1):39-54. Epub 2001/03/17.

41. Amidi M, Mastrobattista E, Jiskoot W, Hennink WE. Chitosan-based delivery systems for protein therapeutics and antigens. Adv Drug Deliv Rev. 2010;62(1):59-82. Epub 2009/11/21.

42. Pawar D, Goyal AK, Mangal S, Mishra N, Vaidya B, Tiwari S, et al. Evaluation of mucoadhesive PLGA microparticles for nasal immunization. AAPS J. 2010;12(2):130-7. Epub 2010/01/16.

43. M.EI-Sherbiny I, Villanueva DG, Herrera D, Smyth HDC. Controlled Pulmonary Drug Delivery. New York: Springer Science+Business Media; 2011.

44. Mert O, Lai SK, Ensign L, Yang M, Wang YY, Wood J, et al. A poly(ethylene glycol)-based surfactant for formulation of drug-loaded mucus penetrating particles. J Control Release. 2012;157(3):455-60. Epub 2011/09/14.

45. Edwards DA, Hanes J, Caponetti G, Hrkach J, Ben-Jebria A, Eskew ML, et al. Large porous particles for pulmonary drug delivery. Science. 1997;276(5320):1868-71. Epub 1997/06/20.

46. Edwards DA, Ben-Jebria A, Langer R. Recent advances in pulmonary drug delivery using large, porous inhaled particles. J Appl Physiol. 1998;85(2):379-85. Epub 1998/08/04.

47. Meenach SA, Kim YJ, Kauffman KJ, Kanthamneni N, Bachelder EM, Ainslie KM. Synthesis, optimization, and characterization of camptothecin-loaded acetalated dextran porous microparticles for pulmonary delivery. Mol Pharm. 2012;9(2):290-8.

Epub 2011/12/14.

48. Gupta V, Davis M, Hope-Weeks LJ, Ahsan F. PLGA microparticles encapsulating prostaglandin E1-hydroxypropyl-beta-cyclodextrin (PGE1-HPbetaCD) complex for the treatment of pulmonary arterial hypertension (PAH). Pharm Res. 2011;28(7):1733-49. Epub 2011/06/01.

49. Surendrakumar K, Martyn GP, Hodgers EC, Jansen M, Blair JA. Sustained release of insulin from sodium hyaluronate based dry powder formulations after pulmonary delivery to beagle dogs. J Control Release. 2003;91(3):385-94. Epub 2003/08/23.

50. Poonam Sheth PBM. Controlled Pulmonary Drug Delivery. New York: Springer Science+Business Media; 2011.

51. Mansour HM, Rhee YS, Wu X. Nanomedicine in pulmonary delivery. Int J Nanomedicine. 2009;4:299-319. Epub 2010/01/08.

52. Sung JC, Pulliam BL, Edwards DA. Nanoparticles for drug delivery to the lungs. Trends Biotechnol. 2007;25(12):563-70. Epub 2007/11/13.

53. El-Sherbiny IM, Smyth HD. Smart Magnetically Responsive Hydrogel Nanoparticles Prepared by a Novel Aerosol-Assisted Method for Biomedical and Drug Delivery Applications. J Nanomater. 2011;2011(2011):1-13. Epub 2011/08/03.

54. El-Sherbiny IM, Smyth HD. Biodegradable nano-micro carrier systems for sustained pulmonary drug delivery: (I) self-assembled nanoparticles encapsulated in respirable/swellable semi-IPN microspheres. Int J Pharm. 2010;395(1-2):132-41. Epub 2010/06/29.

55. Chow AH, Tong HH, Chattopadhyay P, Shekunov BY. Particle engineering for

pulmonary drug delivery. Pharm Res. 2007;24(3):411-37. Epub 2007/01/25.

56. Rytting E, Nguyen J, Wang X, Kissel T. Biodegradable polymeric nanocarriers for pulmonary drug delivery. Expert Opin Drug Deliv. 2008;5(6):629-39. Epub 2008/06/06.

57. Ehrhardt C, Fiegel J, Fuchs S, Abu-Dahab R, Schaefer UF, Hanes J, et al. Drug absorption by the respiratory mucosa: cell culture models and particulate drug carriers. J Aerosol Med. 2002;15(2):131-9. Epub 2002/08/20.

58. Tsifansky MD, Yeo Y, Evgenov OV, Bellas E, Benjamin J, Kohane DS. Microparticles for inhalational delivery of antipseudomonal antibiotics. AAPS J. 2008;10(2):254-60. Epub 2008/05/06.

59. Yang Y, Bajaj N, Xu P, Ohn K, Tsifansky MD, Yeo Y. Development of highly porous large PLGA microparticles for pulmonary drug delivery. Biomaterials. 2009;30(10):1947-53. Epub 2009/01/13.

60. Fu J, Fiegel J, Krauland E, Hanes J. New polymeric carriers for controlled drug delivery following inhalation or injection. Biomaterials. 2002;23(22):4425-33. Epub 2002/09/11.

61. Sanjar S, Matthews J. Treating systemic diseases via the lung. J Aerosol Med. 2001;14 Suppl 1:S51-8. Epub 2001/06/27.

62. P. Sheth. Controlled Pulmonary Drug Delivery. New York: Springer Science+Business Media; 2011. 244 p.

63. Brannon-Peppas L. Med. Plastics. Biomater. Med Plastics Biomater. 1997;4:34-44.

64. Louey MD G-CL. Controlled release products for respiratory delivery. Am

Pharm Rev. 2004;7:82-7.

65. Peiris JS, Guan Y, Yuen KY. Severe acute respiratory syndrome. Nat Med. 2004;10(12 Suppl):S88-97. Epub 2004/12/04.

66. Gencay M, Roth M, Christ-Crain M, Mueller B, Tamm M, Stolz D. Single and multiple viral infections in lower respiratory tract infection. Respiration. 2010;80(6):560-7. Epub 2010/09/24.

67. Akinloye OM, Ronkko E, Savolainen-Kopra C, Ziegler T, Iwalokun BA, Deji-Agboola MA, et al. Specific viruses detected in nigerian children in association with acute respiratory disease. J Trop Med. 2011;2011:690286. Epub 2011/10/19.

68. Linsuwanon P, Payungporn S, Samransamruajkit R, Posuwan N, Makkoch J, Theanboonlers A, et al. High prevalence of human rhinovirus C infection in Thai children with acute lower respiratory tract disease. J Infect. 2009;59(2):115-21. Epub 2009/06/27.

69. Winther B. Rhinovirus infections in the upper airway. Proc Am Thorac Soc. 2011;8(1):79-89. Epub 2011/03/03.

70. Glueck R. Review of intranasal influenza vaccine. Adv Drug Deliv Rev. 2001;51(1-3):203-11. Epub 2001/08/23.

71. Giri PK, Khuller GK. Is intranasal vaccination a feasible solution for tuberculosis? Expert Rev Vaccines. 2008;7(9):1341-56. Epub 2008/11/05.

72. Zaman M, Simerska P, Toth I. Synthetic polyacrylate polymers as particulate intranasal vaccine delivery systems for the induction of mucosal immune response. Curr Drug Deliv. 2010;7(2):118-24. Epub 2010/02/18.

73. Sharma S, Mukkur TK, Benson HA, Chen Y. Pharmaceutical aspects of intranasal

delivery of vaccines using particulate systems. J Pharm Sci. 2009;98(3):812-43. Epub 2008/07/29.

74. Menzel M, Muellinger B, Weber N, Haeussinger K, Ziegler-Heitbrock L. Inhalative vaccination with pneumococcal polysaccharide in healthy volunteers. Vaccine. 2005;23(43):5113-9. Epub 2005/07/21.

75. Gordon SB, Malamba R, Mthunthama N, Jarman ER, Jambo K, Jere K, et al. Inhaled delivery of 23-valent pneumococcal polysaccharide vaccine does not result in enhanced pulmonary mucosal immunoglobulin responses. Vaccine. 2008;26(42):5400-6. Epub 2008/08/19.

76. Vujanic A, Wee JL, Snibson KJ, Edwards S, Pearse M, Quinn C, et al. Combined mucosal and systemic immunity following pulmonary delivery of ISCOMATRIX adjuvanted recombinant antigens. Vaccine. 2010;28(14):2593-7. Epub 2010/01/26.

77. Minne A, Louahed J, Mehauden S, Baras B, Renauld JC, Vanbever R. The delivery site of a monovalent influenza vaccine within the respiratory tract impacts on the immune response. Immunology. 2007;122(3):316-25. Epub 2007/05/25.

78. Kim DY, Sato A, Fukuyama S, Sagara H, Nagatake T, Kong IG, et al. The airway antigen sampling system: respiratory M cells as an alternative gateway for inhaled antigens. J Immunol. 2011;186(7):4253-62. Epub 2011/03/02.

79. Lu D, Hickey AJ. Pulmonary vaccine delivery. Expert Rev Vaccines. 2007;6(2):213-26. Epub 2007/04/06.

80. Florindo HF, Pandit S, Goncalves LM, Alpar HO, Almeida AJ. New approach on the development of a mucosal vaccine against strangles: Systemic and mucosal immune

responses in a mouse model. Vaccine. 2009;27(8):1230-41. Epub 2008/12/31.

81. Muttil P, Prego C, Garcia-Contreras L, Pulliam B, Fallon JK, Wang C, et al. Immunization of guinea pigs with novel hepatitis B antigen as nanoparticle aggregate powders administered by the pulmonary route. AAPS J. 2010;12(3):330-7. Epub 2010/04/27.

82. Thomas C, Gupta V, Ahsan F. Particle size influences the immune response produced by hepatitis B vaccine formulated in inhalable particles. Pharm Res. 2010;27(5):905-19. Epub 2010/03/17.

83. Bivas-Benita M, Lin MY, Bal SM, van Meijgaarden KE, Franken KL, Friggen AH, et al. Pulmonary delivery of DNA encoding Mycobacterium tuberculosis latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice. Vaccine. 2009;27(30):4010-7. Epub 2009/04/25.

84. Thomas C, Rawat A, Hope-Weeks L, Ahsan F. Aerosolized PLA and PLGA nanoparticles enhance humoral, mucosal and cytokine responses to hepatitis B vaccine. Mol Pharm. 2011;8(2):405-15. Epub 2010/12/30.

85. Lemke CD, Graham JB, Geary SM, Zamba G, Lubaroff DM, Salem AK. Chitosan is a surprising negative modulator of cytotoxic CD8+ T cell responses elicited by adenovirus cancer vaccines. Mol Pharm. 2011;8(5):1652-61. Epub 2011/07/26.

86. Heyder J. Deposition of inhaled particles in the human respiratory tract and consequences for regional targeting in respiratory drug delivery. Proc Am Thorac Soc. 2004;1(4):315-20. Epub 2005/08/23.

87. Londahl J, Pagels J, Boman C, Swietlicki E, Massling A, Rissler J, et al. Deposition of biomass combustion aerosol particles in the human respiratory tract. Inhal Toxicol. 2008;20(10):923-33. Epub 2008/08/01.

88. Hofmann W. Modelling inhaled particle deposition in the human lung—A review. Journal of Aerosol Science. 2011;42(10):693-724.

89. Crowder TM, Rosati JA, Schroeter JD, Hickey AJ, Martonen TB. Fundamental effects of particle morphology on lung delivery: predictions of Stokes' law and the particular relevance to dry powder inhaler formulation and development. Pharm Res. 2002;19(3):239-45. Epub 2002/04/06.

90. Hassan MS, Lau RW. Effect of particle shape on dry particle inhalation: study of flowability, aerosolization, and deposition properties. AAPS PharmSciTech. 2009;10(4):1252-62. Epub 2009/10/30.

91. Musante CJ, Schroeter JD, Rosati JA, Crowder TM, Hickey AJ, Martonen TB. Factors affecting the deposition of inhaled porous drug particles. J Pharm Sci. 2002;91(7):1590-600. Epub 2002/07/13.

92. Glover W, Chan HK, Eberl S, Daviskas E, Verschuer J. Effect of particle size of dry powder mannitol on the lung deposition in healthy volunteers. Int J Pharm. 2008;349(1-2):314-22. Epub 2007/10/02.

93. Kim H, Lee J, Kim TH, Lee ES, Oh KT, Lee DH, et al. Albumin-coated porous hollow poly(lactic-co-glycolic acid) microparticles bound with palmityl-acylated exendin-4 as a long-acting inhalation delivery system for the treatment of diabetes. Pharm Res. 2011;28(8):2008-19. Epub 2011/04/08.

94. Steimer A, Haltner E, Lehr CM. Cell culture models of the respiratory tract relevant to pulmonary drug delivery. J Aerosol Med. 2005;18(2):137-82. Epub 2005/06/22.

95. McGill SL, Smyth HD. Disruption of the mucus barrier by topically applied exogenous particles. Mol Pharm. 2010;7(6):2280-8. Epub 2010/10/06.

96. Semmler M, Seitz J, Erbe F, Mayer P, Heyder J, Oberdorster G, et al. Long-term clearance kinetics of inhaled ultrafine insoluble iridium particles from the rat lung, including transient translocation into secondary organs. Inhal Toxicol. 2004;16(6-7):453-9. Epub 2004/06/19.

97. Edsbacker S, Wollmer P, Selroos O, Borgstrom L, Olsson B, Ingelf J. Do airway clearance mechanisms influence the local and systemic effects of inhaled corticosteroids? Pulm Pharmacol Ther. 2008;21(2):247-58. Epub 2007/10/24.

98. Olsson B, Bondesson E, Borgstrom L, Edsbacker S, Eirefelt S, Ekelund K, Gustavasson L, Hegelund-Myrback T. Pulmonary drug metabolism, clearance and absorption. Smyth HDC, Hickey AJ, editors. New York: Springer 2011.

99. Houtmeyers E, Gosselink R, Gayan-Ramirez G, Decramer M. Regulation of mucociliary clearance in health and disease. Eur Respir J. 1999;13(5):1177-88. Epub 1999/07/22.

100. Wang JJ, Zeng ZW, Xiao RZ, Xie T, Zhou GL, Zhan XR, et al. Recent advances of chitosan nanoparticles as drug carriers. Int J Nanomedicine. 2011;6:765-74. Epub 2011/05/19.

101. Nagpal K, Singh SK, Mishra DN. Chitosan nanoparticles: a promising system in

novel drug delivery. Chem Pharm Bull (Tokyo). 2010;58(11):1423-30. Epub 2010/11/05.

102. Park JH, Saravanakumar G, Kim K, Kwon IC. Targeted delivery of low molecular drugs using chitosan and its derivatives. Adv Drug Deliv Rev. 2010;62(1):28-41. Epub 2009/10/31.

103. Duceppe N, Tabrizian M. Advances in using chitosan-based nanoparticles for in vitro and in vivo drug and gene delivery. Expert Opin Drug Deliv. 2010;7(10):1191-207. Epub 2010/09/15.

104. Panos I, Acosta N, Heras A. New drug delivery systems based on chitosan. Curr Drug Discov Technol. 2008;5(4):333-41. Epub 2008/12/17.

105. Gunbeyaz M, Faraji A, Ozkul A, Purali N, Senel S. Chitosan based delivery systems for mucosal immunization against bovine herpesvirus 1 (BHV-1). Eur J Pharm Sci. 2010;41(3-4):531-45. Epub 2010/08/31.

106. Gordon SB, Read RC. Macrophage defences against respiratory tract infections. Br Med Bull. 2002;61:45-61. Epub 2002/05/09.

107. Patton JS, Byron PR. Inhaling medicines: delivering drugs to the body through the lungs. Nat Rev Drug Discov. 2007;6(1):67-74. Epub 2006/12/30.

108. Parra SC, Burnette R, Price HP, Takaro T. Zonal distribution of alveolar macrophages, type II pneumonocytes, and alveolar septal connective tissue gaps in adult human lungs. Am Rev Respir Dis. 1986;133(5):908-12. Epub 1986/05/01.

109. Lombry C, Edwards DA, Preat V, Vanbever R. Alveolar macrophages are a primary barrier to pulmonary absorption of macromolecules. Am J Physiol Lung Cell Mol Physiol. 2004;286(5):L1002-8. Epub 2003/12/26.

110. Fernandes CA, Vanbever R. Preclinical models for pulmonary drug delivery. Expert Opin Drug Deliv. 2009;6(11):1231-45. Epub 2009/10/27.

111. J Ducreux RV. Crucial biopharmaceutical issues facing macromolecular candidates for inhalation: the role of macrophages in pulmonary protein clearance Respiratory Drug Delivery Europe. 2007;1:31-41.

112. Oberdorster G. Pulmonary effects of inhaled ultrafine particles. Int Arch Occup Environ Health. 2001;74(1):1-8. Epub 2001/02/24.

113. Semmler-Behnke M, Takenaka S, Fertsch S, Wenk A, Seitz J, Mayer P, et al. Efficient elimination of inhaled nanoparticles from the alveolar region: evidence for interstitial uptake and subsequent reentrainment onto airways epithelium. Environ Health Perspect. 2007;115(5):728-33. Epub 2007/05/24.

114. Rawat A, Majumder QH, Ahsan F. Inhalable large porous microspheres of low molecular weight heparin: in vitro and in vivo evaluation. J Control Release. 2008;128(3):224-32. Epub 2008/05/13.

115. Karathanasis E, Bhavane R, Annapragada AV. Glucose-sensing pulmonary delivery of human insulin to the systemic circulation of rats. Int J Nanomedicine. 2007;2(3):501-13. Epub 2007/11/21.

116. Patton JS. Mechanisms of macromolecule absorption by the lungs. Adv Drug Deliv Rev. 1996;19(1):3-36.

117. Gebert A, Pabst R. M cells at locations outside the gut. Semin Immunol. 1999;11(3):165-70. Epub 1999/06/26.

118. Hathaway LJ, Kraehenbuhl JP. The role of M cells in mucosal immunity. Cell Mol

Life Sci. 2000;57(2):323-32. Epub 2000/04/15.

119. Kraehenbuhl JP, Neutra MR. Epithelial M cells: differentiation and function. Annu Rev Cell Dev Biol. 2000;16:301-32. Epub 2000/10/14.

120. Helgeson ME, Chapin SC, Doyle PS. Hydrogel microparticles from lithographic processes: novel materials for fundamental and applied colloid science. Curr Opin Colloid Interface Sci. 2011;16(2):106-17. Epub 2011/04/26.

121. Julie Todoroff RV. Fate of nanomedicines in the lungs. Curr Opin Colloid Interface Sci. 2011;16(3):246-54.

122. Dagar S. Gibaldi's Drug Delivery Systems in Pharmaceutical care. Bethesda, MD: American Society of Health-System Pharmaceutics; 2007.

123. Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER. Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis. 1982;126(2):332-7. Epub 1982/08/01.

124. Mercer RR, Russell ML, Roggli VL, Crapo JD. Cell number and distribution in human and rat airways. Am J Respir Cell Mol Biol. 1994;10(6):613-24. Epub 1994/06/01.

125. Codrons V, Vanderbist F, Ucakar B, Preat V, Vanbever R. Impact of formulation and methods of pulmonary delivery on absorption of parathyroid hormone (1-34) from rat lungs. J Pharm Sci. 2004;93(5):1241-52. Epub 2004/04/07.

126. Lombry C, Bosquillon C, Preat V, Vanbever R. Confocal imaging of rat lungs following intratracheal delivery of dry powders or solutions of fluorescent probes. J Control Release. 2002;83(3):331-41. Epub 2002/10/22.

127. Dershwitz M, Walsh JL, Morishige RJ, Connors PM, Rubsamen RM, Shafer SL, et al. Pharmacokinetics and pharmacodynamics of inhaled versus intravenous morphine in healthy volunteers. Anesthesiology. 2000;93(3):619-28. Epub 2000/09/02.

128. Patton JS, Fishburn CS, Weers JG. The lungs as a portal of entry for systemic drug delivery. Proc Am Thorac Soc. 2004;1(4):338-44. Epub 2005/08/23.

129. Boucher RC, Stutts MJ, Gatzy JT. Regional differences in bioelectric properties and ion flow in excised canine airways. J Appl Physiol. 1981;51(3):706-14. Epub 1981/09/01.

130. Matsukawa Y, Lee VH, Crandall ED, Kim KJ. Size-dependent dextran transport across rat alveolar epithelial cell monolayers. J Pharm Sci. 1997;86(3):305-9. Epub 1997/03/01.

131. Bur M, Huwer H, Lehr CM, Hagen N, Guldbrandt M, Kim KJ, et al. Assessment of transport rates of proteins and peptides across primary human alveolar epithelial cell monolayers. Eur J Pharm Sci. 2006;28(3):196-203. Epub 2006/03/15.

132. Ikehata M, Yumoto R, Nakamura K, Nagai J, Takano M. Comparison of albumin uptake in rat alveolar type II and type I-like epithelial cells in primary culture. Pharm Res. 2008;25(4):913-22. Epub 2007/09/14.

133. John TA, Vogel SM, Minshall RD, Ridge K, Tiruppathi C, Malik AB. Evidence for the role of alveolar epithelial gp60 in active transalveolar albumin transport in the rat lung. J Physiol. 2001;533(Pt 2):547-59. Epub 2001/06/05.

Chapter 2: Hydrogels for controlled pulmonary delivery [1, 2]

2.1 ABSTRACT

A significant number of researchers have focused on pulmonary delivery as an alternative administration route owing to no first pass metabolism, low protease, thin epithelium barrier and large surface area in the lung system. Controlled release in the pulmonary delivery system further reduces loading dose, loading frequency and systemic side effects, and also increases duration of action and patient compliance. Compared to other microparticles used in controlled release pulmonary administration, hydrogels, the three dimensional polymeric matrix networks, were recently investigated as a result of particular swelling and mucoadhesive properties which could help pass pulmonary delivery barriers. This review herein first introduces the controlled release drug delivery to the lung, followed by the summary of currently available approaches for controlled release pulmonary drug delivery. Lastly, the origin, advantages, detailed applications and concerns of hydrogels in pulmonary delivery are discussed.

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2.2 INTRODUCTION OF CONTROLLED RELEASE DRUG DELIVERY TO THE LUNG

Pulmonary delivery is to deliver drug particles directly to the lung through respiratory system. Due to advantages of large surface area for absorption, high permeability and no first pass metabolism, there are a variety of inhaled aerosols currently marketed for respiratory inflammation (1), cystic fibrosis (2), other lung disorders and several in development for rapid, noninvasive, systemic delivery of therapeutic agents. Challenges for pulmonary delivery also exist besides the advantages. Inhaled particles must first deposit into the respiratory systems for the therapeutic effects to occur. Large numbers of factors contribute to particle deposition, including particle size, shape and density, airflow velocity and volume, and the duration between inspiration and expiration. Associated with these factors, the mechanisms governing aerosol particle deposition within the airways are inertial impaction, sedimentation, diffusion and interception (3); while inertial impaction, sedimentation and diffusion are the major mechanisms. Once inhaled, particles firstly deposit on the mucosa covered by lung lining fluid. Particles soluble in the mucosal matrix will rapidly diffuse into the epithelial lining fluid and become available for absorption at different rates depending on their physicochemical properties. However, poorly soluble drugs and drugs deposited as particulate materials have to dissolve before absorption and may undergo natural clearance caused by mucociliary escalator, cough, and/or alveolar macrophage phagocytosis. Insoluble or slowly dissolving particles deposited in the conducting airways are eliminated primarily by the mucociliary clearance, while particles that are small enough to deposit in the alveolar region are likely to be phagocytosed by alveolar

macrophages. These deposition obstacles and clearance mechanisms yield short particle residence times in the airways and therefore resultant clinical/pharmacological effects are often much abbreviated requiring most aerosol medications to be administered multiple times daily.

In addition to the convenience of once a day dosing, controlled release pulmonary drug delivery has numerous potential advantages, including reduced side effects, increased duration of action and improved compliance as well as potential cost savings. Controlled release formulations may allow patients with chronic diseases such as asthma to take treatments less frequently, without compromising the amount of drug patients are required to take into the lung. Recent efforts by companies such as Theravance and GlaxoSmithKline for example, indicate the strong desire to allow once a day dosing with similar clinical outcomes to current therapies (4). In addition, controlled formulations can moderate the drug peaks as to reduce toxicity which is common issue for some immediate release formulations containing drugs of narrow therapeutic indices, since the latter is often reported to cause toxicity and reduced efficacy. Another advantage of controlled delivery systems is evident by controlling delivery of two or more therapeutic agents from a single particle system to the lung. Consequently, a high possibility of increased synergism and additive effects can be observed. Many agents currently delivered to the airways are short acting, have short plasma half-lives, and thus are potential candidates for controlled release pulmonary delivery (5, 6). In recent years, controlled pulmonary delivery system has been so far unexploited but it is becoming increasingly attractive. Notably, there are no controlled release delivery pulmonary formulations currently on the market, although this field has been of interest to researchers for decades. Therefore,

development of sustained, modified, or controlled release therapeutic agents using biodegradable and biocompatible polymers would be beneficial for both local and systematic inhalation therapies.

2.3 BRIEF SUMMARY OF APPROACHES USED TO DATE FOR CONTROLLED RELEASE PULMONARY DRUG DELIVERY

Suitable inhaled carriers for controlled release pulmonary drug delivery should possess adequate aerodynamic properties, drug release, biodegradation and evasion of lung clearance mechanisms including mucociliary escalator and macrophage uptake. There have been a variety of carriers investigated, such as liposomes, biodegradable polymeric microspheres (MS), bioresponsive carriers, prodrugs, co-precipitates and hydrogels amongst others (7). Table 1 summarizes characteristics of commonly studied carriers for controlled release pulmonary drug delivery.

2.3.1 Liposomes

Liposomes are self-assembling lipid bilayers, with hydrophobic inner layer and hydrophilic outer layer. Liposomes can be prepared with a range of size and layers and will encapsulate both hydrophobic and hydrophilic drugs (27-32). Loading doses, drug release dynamics from the liposomes, and membrane properties can be adjusted by changing the compositions and ratio of various lipids (33). The percentage of drug entrapped in the liposomes, as an example, ranges from 55% to 80% and may even reach 90% with different ratio of lipids (6, 12, 13, 34). Liposomes have been the most commonly used and investigated vehicles for controlled release of drugs for pulmonary delivery due to their biocompatibility, safety profile, and ability to avoid induction of an

immune response. Some liposomal pulmonary products have also progressed into advanced animal studies or clinical studies in humans (32, 33, 35).

Liposomal dry powder inhaler formulations typically are prepared by lyophilization from liposome suspensions using different kinds of cryoprotecants in various mass ratios (e.g. sucrose, sorbolac, glutose, mannitol, trehalose or lactose). The function of cryoprotecants is to preserve the structural and functional integrity of liposomes during freeze drying process. But drug encapsulation efficiency after dehydration-rehydration generally will decrease depending on the cryoprotecant type and concentration used(6, 8). The lyophilization process normally takes days and is therefore not cost effective in many cases. The resulting porous cake after lyophilization have to be crushed by ball or jet milling mechanically and/or sieved through #120, #240 or #400-mesh manually, generating irregular and cohesive micron particles with poor flow. The flowability of dry powders is an important factor that influences the *in vitro* deposition performance. The liposomes subjected to lyophilization, milling and sieving therefore need to be mixed with coarse lactose carriers 63-106 μm sieved alpha-lactose monohydrate) to achieve higher fine particle deposition and correspondingly higher fine particle fraction (FPF) for inhalation delivery (6, 9).

One step spray drying is also available to incorporate drug encapsulated liposome suspensions into dry powder inhalers (14, 36). Even spray dried liposomes have narrow size distributions and relatively good flow behavior thus potentially significantly higher inhalation performance without micronization and further mixing with coarse lactose carriers(9, 12, 14), physicochemical characterization indicates that these liposomes are still porous light spherical particles . Therefore, the limitations of liposomal dry powder

are low drug loading, large volume and poor stability whatever manufactures method used. Compared to commercial dry powder inhalers (e.g. Aerolizer and Handihaler), which require size 3 capsules, the liposomal dry powders are filled into size 2 capsules to provide equivalent strength of drugs due to large ratio of inert excipients, cryoprotecants and carriers (6, 12, 14, 15). The physical stability of liposomal formulations may be the major obstacle for commercialization. The guidelines for pharmaceutical acceptable liposomal formulations is 1-2 years stability under room temperature with high drug retention and no significant change in particle shape and size, which may not be easy to satisfy for currently designed liposomal dry formulations (9, 14, 15).

2.3.2 Biodegradable Polymeric Microparticles

Biodegradable microspheres prepared from synthetic polymers have been studied extensively for various administration routes as the characteristics of microspheres allow for both targeted and sustained drug release. Likewise, scientists have researched the use of the synthetic polymers for the sustained release of drugs in the lung and have demonstrated several reasons for the polymers to be used in the lung: a) the compositions of the material are safe in humans for inhalation,; b) the release of therapeutics entrapped could be regulated by varying the monomer compositions, ranging from hours up to several weeks. c) continuous release profiles up to 7 days of drugs with various molecular weights (443 to 5 x 10^6 Da) may be delivered; d) the particle clearance from phagocytosis in the deep lung could be decreased e.g. by addition of PEG to the polymer backbone); e) varying the polymer content changes surface properties and aid aerosolization efficiency to the deep lung; f) polymer can be used to generate highly porous large microparticles

that avoid macrophage clearance without compromising high deposition of drugs throughout the lung (25, 37).

For effective delivery of particles into the lung, physical properties of the particles are crucial, especially the particle size, with the optimal range of 1-5 μm. Microparticles ideal for inhalation are susceptible to rapid alveolar macrophage removal from the lung(38). Typically, particles with aerodynamic diameter smaller than around 5 μm may be delivered to the deep lung. Larger microparticles (e.g. 5-20 μm in diameter) with low density may also be designed such that they have a smaller aerodynamic diameter (< 5 μm) and circumvent phagocytosis by macrophage. These type of engineered large porous microparticles exhibit the aerodynamic size of smaller particles are not only suitable for deep lung delivery, but they also have geometric sizes large enough to resist alveolar macrophage uptake and subsequent clearance prior to drug release from the carrier vehicles (16, 39).

2.3.3 Bioresponsive drug delivery systems

Apart from conventional diffusion controlled release from polymeric delivery systems, bioresponsive drug delivery systems are attractive due to several potential advantages over traditional polymeric drug delivery systems. Drug release from polymeric delivery systems generally happens as the polymer degrades through nonspecific chemical reactions. Due to differences in physiological states and locations of disposition, these passive drug release mechanisms may result in inconsistent drug release profiles, significant intra and inter-patient variability, and poor response to physiological changes in the body (40). While a number of the bioresponsive systems

have been investigated for oral or even parenteral delivery, few literature reports exist for these systems applied to inhalation and the lung (40, 41).

2.3.4 Hydrogels

Hydrogels, the three-dimensional, polymeric matrix networks (42), are composed of water-soluble natural or synthetic polymers, which are cross-linked and absorb a large amount of water into the developed network structure (42, 43). Many hydrogels have these properties and are closely related in terms of physicochemistry to endogenous extracellular matrix present throughout the body. Due to these advantages, hydrogels have been used in many clinical investigations, such as tissue engineering, cellular immobilization, separation of biomolecules or cells, as well as drug delivery (44). In the case of drug delivery, hydrogels are regarded as a potentially ideal delivery system, given their generally high biocompatibility to living tissues with respect to its physical properties, biodegradability, low toxicity, high water content, and low interfacial tension (43). The low interfacial tension with the surrounding biological environment minimizes protein adsorption and adhesion. As a consequence, low adverse interactions of the gel surface with the aqueous biological environment are observed. These observations were first established over 50 years ago (2). In addition, hydrogels are capable of incorporating a variety of molecules for optimized controlled-release profiles, which are controlled by adjusting polymer structure, degree of crosslinking and polymer ratios (42, 43).

Furthermore, controlled drug release hydrogels responsive to external stimulus (e.g. the pH value (45, 46), local tissue temperature (47, 48), electric field (47, 49), magnetic field(50-52), and specific disease-related enzymes (53, 54) are developed. These external conditions can be used as triggers to more specifically control drug release in a “smart”

manner. These bio-responsiveness switches drug release on and off according to spatial or temporal requirements of the drug, thus reducing the frequency of drug administration and the side effects due to off-targeting and undesired body distribution (55, 56).

Given the need to develop controlled drug release systems for pulmonary delivery (57, 58), hydrogel systems have recently been the subject of several investigations reported in the literature. A variety of molecules, such as antibiotics (44), antibodies (59), antitumor agents (60), nanoparticles (61), and even magnetic particles (62), have recently been incorporated into hydrogel systems for direct lung administration. However, in contrast to other routes of administration, studies of hydrogels for pulmonary delivery are less common and are more frequently investigated in oral dosage forms.

With so many carriers systems available for controlled release pulmonary delivery, the focus of this review however is the utilization of hydrogel based systems for pulmonary drug delivery.

2.4 ADVANTAGES OF HYDROGEL-BASED SYSTEM FOR PULMONARY DRUG DELIVERY

In the respiratory system, the conducting airway is covered with a relatively thick layer (5 - 10 μm) of mucus (63, 64), which exhibits specific surface characteristics (such as wettability and adhesivity) to trap exogenous particulates that have been inhaled (65). This mucoadhesive nature endows a direct physical interaction between a solid surface (i.e. inhaled particle containing drug) and mucus layer. The close interaction may significantly increase the bioavailability of a drug due to the longer residence time of drug formulation in the respiratory system, especially if the formulation is made of hydrogel particles which have particular mucoadhesive properties. The increased

contacting time between hydrogel formulation and mucus layer permits a prolonged period for the penetration of drug formulation into mucus layer, thus leading to a potentially higher absorption of drug.

Given that higher bioavailability of drug may be achieved due to mucoadhesive interactions between drug formulation surfaces and mucus layer as shown most extensively in the oral dosage form literature, the development of mucoadhesive systems for pulmonary delivery has been postulated as a potential advantageous method of sustaining drug release. However, as the literature has reported that mucoadhesive particles might inhibit the mucociliary clearance via increasing the viscoelastic properties of the mucosal fluids, there is a deep concern for the time period of mucus renewal and side effects of inhibiting mucociliary clearance (66). In addition to mucociliary clearance, another important clearance mechanism in airway is the rapid macrophage uptake of inhaled particulates (59, 67-69). For macrophage uptake, the preferred geometric size range of particles is overlapped with most respirable aerodynamic size range of 0.5 μm – 5 μm (70, 71). The size issue results in the rapid phagocytosis of drug delivery particles and subsequent loss of pharmacological effect of the therapeutic agents. To address this matter, several approaches have been studied. For example, respirable porous large particles were developed with a larger geometric size (>6μm) as to be beyond the preferred size for macrophage uptake (72-74). Also, there is some evidence that nano-sized particles may effectively evade the macrophage uptake in the alveolar region, because the particles are too small to be recognized by macrophages (75, 76). However, the particles smaller than 0.5 micron, without a prolonged breath hold, are exhaled out of airway (77-80). If nanoparticles are inhaled as microsized aggregates/agglomerates due to

higher cohesive force, nanoparticle deposition in the lung may occur (70, 81, 82). The third method is to fabricate the polymeric hydrogels with dry state. The dried state hydrogels have proper respirable aerodynamic sizes to locate into the deep lung where effective absorption occurs, while swell to large geometric sizes upon exposed to the moist lung epithelia lining fluid, thus prevented from macrophage uptake (83, 84).

Hydrogel particle residence time can be increased in the airways using two main methods: (1) within the conducting airways, or (2) avoidance of macrophage uptake in the deep lung. Particle adhesion to the mucus (mucoadhesion) has widely been exploited as a method to overcome the limitations imposed by mucociliary clearance, especially in the nasal cavity and GI tracts. Here, long polymer chains attached to the surface of particles may enable the particles to become intertwined and entangled with the mucus network. Because of this interaction, mucoadhesive particles typically exhibit slower particle transit time from the mucosal site, likely due to an increase in the viscoelastic properties of the mucosal fluid which inhibits the mucociliary clearance. The ability of mucoadheisve particles to limit clearance in vivo has been indirectly measured in a few cases in the airways (85-87). However, the potential limitations to this strategy include the time scale for mucus renewal and concern over potential negative effects of inhibiting mucociliary clearance.

Avoidance of macrophage uptake in the non-ciliated peripheral airways can be achieved chemically or physically. Chemically, highly hydrated polymers like polyethylene glycol can confer greater levels of stealth to foreign particles. This is well known and deployed heavily by the field of PEGylation. In the lung, the same principles apply. Physically, macrophage engulfment can also be delayed or avoided by ensuring

the particle size (of the swollen hydrogel) is too large for efficient pahgocytosis. Swellable particle avoidance of macrophage uptake is addressed below"

Thus, recent reports have realized two significant properties of hydrogels that may be useful in overcoming some of the barriers of controlled pulmonary drug delivery: (1) the potential for achieving mucus adhesion and (2) the ability to control swelling behavior of the hydrogel particles. Hydrogels with enabled mucus-adhesive properties allow for a reduced clearance from the mucocilliary escalator, leading to longer residence time within the respiratory tract, and higher absorption rate into circular system (43, 88, 89). Swelling, the basic property of hydrogels, can be controlled by the polymer type, crosslinking degree, and processing conditions of hydrogel particles (43, 90-93). Swelling of hydrogels will alter the physical (e.g. particle size distributions) and chemical characteristics (e.g. water content, surface tension) of hydrogels particles to evade alveolar macrophage uptake.

2.5 EXPERIMENTAL APPLICATIONS OF HYDROGELS FOR CONTROLLED PULMONARY DELIVERY

2.5.1 Hydrogel with synthetic polymers

In general, synthetic hydrogels have been studied more often than natural hydrogels due to their well-defined molecular weight and designable modification. Also, the interaction between cells and these synthetic hydrogels are closely influenced by surface mechanical characterization of hydrogels (94-96). In a report (97), a copolymer hydrogel composed of 2-hydroxyethyl methacrylate (HEMA) and 2-methacryloxyethyl trimethyl ammonium chloride (MAETAC) was exploited to study the pulmonary artery endothelial cells attachment. Increasing proportions of positively charged MAETAC increased the

swollen states of hydrogel, as well as its positive charge on surface. Hydrogel with more positive charge promoted the pulmonary artery endothelial cells attachment. This result indicated that surface charge density of hydrogel is a critical factor for the binding interaction between hydrogel and cells, especially for the lung targeted delivery.

Hydrogel-based polyacrylamide microparticles (PMP, 1-5 μM) and nanoparticles (PNP, <100 nm, diameter) was formed (98). These hydrogels were intratracheally administered to mice to investigate the lung retention, cell-uptake, and clearance. These investigations focused on interactions of the hydrogel particles of different size and surface modification with respiratory environment, epithelial and immune cells, and extrapulmonary organs. Results from the in vivo lung retention study showed that nanoparticles had better lung tissue location and retention than microparticles, and nanoparticles were mainly cleared via microphage uptake. . In addition, the author found that the clearance of microparticle, at one side, was accomplished by mucociliary activity, which was evident by the higher biodistribution than nanopartilces, especially in GI tract, such as stomach and intestine. Also, at the other side, microparticles would penetrate into the systemic circulation by transit through the alveolar epithelium-capillary endothelial barriers, which could be related to some unrecognized alteration or injury to the alveolar epithelial cell basement membrane as the author mentioned. In this study, the author investigated the effect of hydrogel'size on their fates in the animal study, showing that synthetic polymer-based hydrogel particles either in micro-size or nano-szie, could be untilized to provide higher local concentration of drug or imaging agents, limite systemic toxicity, and reduce dose frequency.

2.5.2 Hydrogel with natural polymers

A novel Zn^{2+}-cross-linked alginate microparticles for controlled pulmonary delivery of protein drugs was developed (99). The approach to fabricate microparticles was accomplished by one-step spray drying aqueous alginate solutions, containing the BSA, $Zn(NH_3)_4SO_4$, and other additional excipients. Via spray drying, water and NH_3 were evaporated, leaving the free cations of Zn^{2+} to physically interact with the negative charged alginate chain.

The in vitro release study was conducted to investigate the underlying protein release mechanism from this novel Zn^{2+}-cross-linked alginate microparticles, as well as the influence of the type of releasing medium on protein release and physical structure of aliginate microparticles. The result showed that the model protein, BSA, had the slowest release profile from the particles composed of BSA, aliginate and $Zn(NH_3)_4SO_4$, than that from the controlled groups, including particles pure BSA, particles of BSA and alginate, and particles of BSA and $Zn(NH_3)_4SO_4$. The appropriate explanation for the observed slowest release profile could be attributed to two barriers hindering the BSA release. Firstly, BSA should penetrate through tight hydrogel network formed by the Zn^{2+} and alginate. Secondly, it was observed that a complex of znic and BSA was formed in this formulation, which indicates BSA also needs to get free from this complex before being released. Not only had the presence of Zn^{2+} in the cross-linked and protein complex influence the release profile, but the components and their concentrations in the release medium also largely affected the protein release with respect to the erosion of Zn^{2+} cross-linked alginate networks. For example, 5-fold-diluted USP 34 phosphate buffer had a slower release than that of un-diluted buffer, since the latter had a higher concentration of

cations which will compete with Zn^{2+} for the binding site in alginate chains. Additionally, white precipitate consisting of needle-shaped $Zn^{2+}(PO_4)_2$ crystals observed in the release medium accelerated the erosion of alginate networks.

Carrageenans, natural sulfate polysaccharides, have been formed into hydrogel and applied widely in biomedical research (100-104). This is because carrageenan hydrogel has the thermos-reversibility and appropriate viscoelastic properties. In its application of drug delivery systems, carrageenan hydrogel has been served as drug carriers for meloxicam (105), β-carotene (106), camptothecin (107) and ketoprofen (108). Even no carrageenan hydrogel was reported in pulmonary delivery till now; carrageenan had been exploited as a viscous vehicle on pulmonary absorption of antiasthmatic drugs, theophylline and fluticasone propionate (109). Pulmonary absorption of these two drugs was investigated in rats. In vivo results showed that, among the three classes of carrageenan, which are kappa, iota, and lambda, iota-carrageenan could significantly decrease the Cmax of theophylline, while increase its Tmax value. In case of kappa-carrageenan, it could also regulate the pulmonary absorption of fluticasone propionate. These results revealed that carrageenan is an appropriate candidate for pulmonary formulation; its sub-components could even be utilized for controlling delivery of a certain drug.

Gelatins are obtained from animal collagens by either acid hydrolysis (type A) or alkaline hydrolysis (type B). Because of the highly biocompatibility and, gelatin has been widely applied in pharmaceutical formulation, such as implantable delivery system as a biodegradable matrix materials (110), as well as hard or soft gelatin capsules (111, 112). In the application of pulmonary delivery, gelatin was formed as microsphere to deliver

salmon calcitonin (113). Two types of gelatin microspheres were formulated using the difference of isoelectric point between acidic gelatin (5.0) and basic gelatin at pH 7.0. Acidic gelatin expressing negative charge released 40% of salmon calcitonin within 2 h, which is much slower than that from the basic gelatin showing positive charge, because the positive charge formulation showed a cumulative release of almost 85% within same period. Both of these two formulations were administered into rats via pulmonary route to investigate their bioactivity compared to pure drug in PBS solution. These *in vivo* studies revealed that positively charged gelatin microspheres with smaller size of 3.4μm appeared to have better effect than negatively charge gelatin formulation with a size around to 10 μm; however, in contrast to control group of pure drug in PBS solution, gelatin formulation indicated an improved and sustained bioactivity of salmon calcitonin. This study demonstrated the capability of gelatin to deliver drugs via pulmonary route in a controlled manner.

Hyaluronic acid (HA) is a naturally occurred polymer which has been well studied in biomedical application due to its high biocompatibility and low immunogenicity (114-117). In its application of pulmonary drug delivery, several advantages had been highlighted for utilizing HA as a drug carrier (113). Firstly, HA is endogenous to the pulmonary environment. In addition, it regulates the function of various inflammatory mediators. Finally, the bio-adhesive property allows HA formulation achieve a longer retention period in respiratory by avoiding the mucociliary clearance (113). Given these mentioned benefits, several non-hydrogel formulations composed of HA have been developed for delivery medicines to the lung, such as insulin (118) and fluticasone propionate (113). Studies showed that effective delivery manner of these therapeutic

agents was observed form these HA formulations which are not in HA hydrogel forms. Even though no HA hydrogel formulations designed specifically for pulmonary delivery have been investigated, HA hydrogels are well practiced approaches for drug delivery (116, 119-121), and its application of incorporating medications for lung delivery is a promising topic for investigation.

The fate of hydrogels, either with synthetic polymers or natural polymers, such as carrageenans and gelatin, depends on the deposition site in the lung just the same as other particles. Generally, particles deposited in the conducting airway are cleared by the mucociliary escalator and then are transported to GI tract. Specifically, particles entrapped within the mucus and macrophages are carried up with mucociliary escalator to the pharynx for swallow.

On the other hand, particles deposited in the alveolar region are phagocytosed by macrophages. These macrophages may arrive at the terminal bronchioles, also get access to mucociliay escalator, and then are cleared from the body. Other macrophages or inflammatory cells can carry the phagocytosed particles into the interstitial spaces. After entering into the interstitial space, the particles in the macrophages are transported to the regional lymph modes through the lymphatics or may go directly to the bloodstream.

Besides, carrageenans and gelatin may be enzymatically degraded. Enzymes found in the intestinal-hepatic metabolism system are also observed in lungs but with lower level, therefore the drug-metabolizing capability of the lung is lower than that of the liver. In addition, there is a possibility that these exogenous components can trigger the inflammatory response in the lungs.

2.5.3 Hydrogel in a nanoparticle-in-microgel form

Recently, an innovative nanoparticle-in-microgel system was development for pulmonary delivery (59). This unique system simply composed of a multi-armed poly (ethylene glycol) crossed linked with trypsin sensitive peptide. The cross-linking reaction was achieved through a newly developed method, involving a Michael addition during (water-in-oil) emulsion (MADE). With only one additional procedure of mixing, this fabricated microgel system could incorporate nanoparticles with a wide size range of 20nm to 200nm, and also encapsulated some biological agents, such as IgG and DNA as examples. This microgel was investigated with the aim of overcoming the critical challenges for pulmonary delivery, including efficient deep lung delivery, avoiding rapid clearance by macrophages, as well as disease-specific triggered release. Effective delivery of inhalable particles to deep lung region requires that the aerodynamic diameter of particle should be in the range of 0.5-5 μm (83, 122). The normalized theoretical aerodynamic diameter over the entire population for these microgel particles was about 4.809 μm, allowing sufficient deep lung delivery. The aerodynamic diameter of inhalable particles was calculated based on its geometric diameter, which was about 4.694 μm in this case. After 24 hrs of swelling, the geometric size of microgel was larger than 6 μm, which is beyond the preferred size for rapid macrophage uptake. However, the period for the uptake of micro-sized particles by macrophage is within mins (123-125), 24 hrs is obvious longer for the microgel to avoid the uptake. Nonetheless, the microgel developed in this study which was freshly made (pre-swelling) could effectively avoid macrophage uptake at both 2 and 24 h. This interesting phenomenon could partially attribute to the hydrophilic materials, swelling behavior, as well as PEG composition. After avoiding

macrophages uptake, microgel would rapidly release its biologics or nanoparticles in the presence of specific enzyme under a disease-responsive drug release mechanism. This disease–stimuli release manner could largely improve the drug potency and targeting efficiency, while reducing side effects because of non-uniform distribution in the respiratory system.

2.5.4 Dried swellable hydrogel particles

Acknowledging the current barriers to successful pulmonary delivery, various approaches have been reported in literatures as to improve the deep lung targeting, to avoid the efficient clearance, as well as to achieve a sustained/controlled release profile. These approaches in terms of formulations could be summarized as nanoparticles, microencapsulated particles, PEGylated drugs, large porous particles, and micro/nano-microparticles systems. Our group has consistently focused on the development of dried swellable hydrogel particles (60, 83, 84, 126). We proposed that formulations typically designed for pulmonary drug delivery should exhibit the characterization of dried hydrogel drug carriers, which have respirable aerodynamic size and escape from uptaking by macrophases, in addition to showing desirable controlled release kinetics. These dried hydrogel carrier systems were successfully developed, which, in the dehydrated state, exhibit appropriate aerodynamic profile for delivering to deep lung region; after deposition in the hydrated respiratory tract, the dried hydrogels gradually swell to a larger size within minutes, thus leading to evade macrophage uptake and release of encapsulated therapeutic agents, either nanoparticles or molecules in the swelled particles (61, 126). One of recent formulations exploited in our lab is to incorporate drug-loaded nanoparticles into swellable/respirable microparticles, with the aim of achieving a

sustained drug release (126). This nano-in-microparticles formulation would release the majority drug under a controlled manner in the target region where the uptake of micro-size particles by macrophages takes place. In this work, the target region was the alveolar region. Additionally, the nanoparticles fully encapsulated into the microparticles matrix would not be exhaled from the lung after inspiration. The encapsulated nanoparticles were first fabricated using poly (D, L-lactic-co-glycolic acid) (PLGA) to load curcumin, with a modified single emulsion-solvent evaporation method. Then, the drug-loaded nanoparticles were added into the solution of PEGylated Chitosan to form a homogeneous mixture, which were spray dried to obtain the respirable/swellable hydrogel microspheres. After a series of in vitro characterizations, the average particle geometric size for drug- loaded PLGA nanoparticles and hydrogel microspheres encapsulating the drug-loaded PLGA nanoparticles were around 230 nm and 3.5 μm, respectively. Prepared formulations showed a fast initial swelling within the first few minutes. For example, the diameter of particles had increased from about 3−3.5 μm when dry to 35 μm after 6 min of swelling. This swelling continued regularly with time to reach 80 μm at 20 min. To avoid macrophage clearance, microparticles must typically have a diameter >6 μm. In addition, the mass median aerodynamic diameters (MMAD, μm) of the inhalable hydrogel microspheres were in the range of 1.25-1.96 μm. This hydrogel with ideal aerodynamic diameter showed two phase of drug release in the in vitro test. Initially, 20% of curcumin was released within the first hour, which followed with a slow release manner of releasing 45% of drug by 24 hrs. The fast initial release was largely contributed to the swollen of hydrogel upon interacting with release medium during the first 1 hr.

2.6 CONSIDERATION OF THERAPEUTIC DOSAGE FOR CONTROLLED PULMONARY DELIVERY

Compared to immediate release formulation, controlled release formulations are generally designed to contain multiple doses, which provides therapeutic effects over a longer period with fewer administration frequency. In addition, controlled release formulations for inhalation are becoming increasingly attractive due to the advantages of respiratory systems for the local and systemic drug delivery. However, till now there is no controlled release inhalation dosage form approved in the USA. The absence of which may be the result of the untested long-term safety profile of many of the excipients that could be potentially used (127-129).

Generally, it is the excipients which control and contribute the most to the prolonged drug release. Thus, a large mass fraction of excipients in the sustained release dosage from may be administered daily through inhalation pathway. Furthermore, the excipients used in controlled dosage forms are often polymers, either from natural or synthetic resources. However, few polymers are in approved inhalation products. Therefore, the usage of un-approved excipients in the controlled release formulation for inhalation will require extensive toxicity testing.

One important consideration in the development of controlled or sustained release formulations in pulmonary drug delivery is the dose. Because at least a 24 hour dose might seem a basic requirement for these systems one must consider the mass of drug required to be loaded into the delivery system to maintain therapeutic levels during this period. In addition, the mass of the delivery system must also be considered because there appear to be limits on the total mass of aerosol that can be administered. Typical DPIs

deliver between 18 and 550 µg of active drug per dose (130). In addition, there is signficant clinical experience with larger powder doses to the airways. The recommended dose of Bronchitol, for example is 400 mg. This requires the inhalation of the contents of ten capsules via the inhaler device. Also, the Tobi(R) Podhaler (TM), delivers 112 mg of powder to the airways using four 28 mg inhalations. Therefore if one can estimate the order of magnitude limits of drug loading in the sustained release matrix as being as approximately 1% w/w in dry powder systems. If however, large payloads of drug are required to be delivered, as in the case of antibiotics in the treatment of lung infections, much higher drug loadings will be required, or the order of 50% or more.

2.7 FUTURE PERSPECTIVE

Intensive studies in hydrogels have contributed our present understanding of this unique delivery formulation. Due to its properties, such as mucoadheisive and swellable, hydrogel seems to be a promising strategy for pulmonary drug delivery. In addition, a large number of therapeutic agents, including small molecules, peptides, and protein, even gene, could be incorporated into the polymeric matrix of hydrogel, which significantly enlarged the scope of disease treated via respiratory delivery. Moreover, the controlled release manner of these therapeutic agents, as well as their specific site targeting, largely reduced the side effects and administration frequency. However, a huge gap between the experimental data and clinical reality still exists due to critical challenges in terms of the physiological variations within different respiratory diseases, location of deep lung, less immunological response, and long term usage safety. Therefore, further studies of both experimental and clinical aspects have to be explored to reveal the potential of hydrogel application in pulmonary controlled drug delivery system.

2.8 TABLE

Table 2.1 Types of Commonly Investigated Carriers for Controlled Release Pulmonary Delivery

Delivery System	**Characteristics**	**Materials**	**Ref**
Liposomes	(a) Amphiphilic (b) Biocompatible and biodegradable (c)Targeting potential and ease of functional modification (d) Tolerable and safe	egg phosphatidyl choline and cholesterol, soybean phosphatidylcholine (SPC), Hydrogenated soyaphos-phatidylcholine (HSPC) and hydrogenated soyaphosphatidyl-glycerol (SPG-3)	(6, 8-15)
Biodegradable polymeric microparticles	(a)Biocompatible and biodegradable (b)Slower release rate and longer duration of action, release rate controllable (c)Decreased particle clearance from phagocytosis	Albumin, sebacic acid (SA) Poly(ether-anhydrides) poly(lactide and/or glycolide), Poly(lactic-co-glycolic acid) (PLGA) poly (ethylene glycol) (PEG) 1,3-bis(carboxyphenoxy)propane (CPP)	(7, 16-25)
Hydrogel	(1) Swellable, (2) Evade alveolar macrophage uptake; (3) Biocompatible and low toxicity;	Natural polymers: Chitosan, Alginate, Collagen, Gelatin, Hyaluronic acid and Dextran Synthetic polymers: Hydroxyethylmethacryate (HEMA), Vinyl acctate (VAc), N-(2-Hydroxy propyl)methacrylate (HPMA)	(26)

2.9 REFERENCES

1. Einarsson O, Geba GP, Zhou Z, Landry ML, Panettieri RA, Jr., Tristram D, et al. Interleukin-11 in respiratory inflammation. Annals of the New York Academy of Sciences. 1995;762:89-100; discussion -1. Epub 1995/07/21.

2. Niven RW. Delivery of biotherapeutics by inhalation aerosol. Critical reviews in therapeutic drug carrier systems. 1995;12(2-3):151-231. Epub 1995/01/01.

3. Balashazy I, Hofmann W, Farkas A, Madas BG. Three-dimensional model for aerosol transport and deposition in expanding and contracting alveoli. Inhalation toxicology. 2008;20(6):611-21. Epub 2008/04/30.

4. Theravance. Theravance Reports Fourth Quarter and Full Year 2012 Financial Results. Theravance, Inc. 2013.

5. Cook RO, Pannu RK, Kellaway IW. Novel sustained release microspheres for pulmonary drug delivery. Journal of controlled release : official journal of the Controlled Release Society. 2005;104(1):79-90. Epub 2005/05/04.

6. Huang WH, Yang ZJ, Wu H, Wong YF, Zhao ZZ, Liu L. Development of liposomal salbutamol sulfate dry powder inhaler formulation. Biological & pharmaceutical bulletin. 2010;33(3):512-7. Epub 2010/03/02.

7. Zeng X, Martin G, Marriott C. The controlled delivery of drugs to the lung. International journal of pharmaceutics. 1995;124(2, 3):149-65.

8. Joshi MR, Misra A. Liposomal budesonide for dry powder inhaler: preparation and stabilization. AAPS PharmSciTech. 2001;2(4):25. Epub 2004/01/20.

9. Shah SP, Misra A. Development of liposomal amphotericin B dry powder inhaler formulation. Drug delivery. 2004;11(4):247-53. Epub 2004/09/17.

10. Bi R, Shao W, Wang Q, Zhang N. Spray-freeze-dried dry powder inhalation of insulin-loaded liposomes for enhanced pulmonary delivery. Journal of drug targeting. 2008;16(9):639-48. Epub 2008/11/05.

11. Chen KH, Mueannoom W, Gaisford S, Kett VL. Investigation into the effect of varying l-leucine concentration on the product characteristics of spray-dried liposome powders. The Journal of pharmacy and pharmacology. 2012;64(10):1412-24. Epub 2012/09/05.

12. Chougule M, Padhi B, Misra A. Development of spray dried liposomal dry powder inhaler of Dapsone. AAPS PharmSciTech. 2008;9(1):47-53. Epub 2008/05/01.

13. Chougule MB, Padhi BK, Misra A. Nano-liposomal dry powder inhaler of Amiloride Hydrochloride. Journal of nanoscience and nanotechnology. 2006;6(9-10):3001-9. Epub 2006/10/20.

14. Mahavir Chougule, Bijay Padhi, Misra A. Nano-liposomal dry powder inhaler of tacrolimus: Preparation, characterization, and pulmonary pharmacokinetics. International Journal of Nanomedicine. 2007;2(4):675-88.

15. Shahiwala A, Misra A. A preliminary pharmacokinetic study of liposomal leuprolide dry powder inhaler: a technical note. AAPS PharmSciTech. 2005;6(3):E482-6. Epub 2005/12/16.

16. Arnold MM, Gorman EM, Schieber LJ, Munson EJ, Berkland C. NanoCipro encapsulation in monodisperse large porous PLGA microparticles. Journal of controlled

release : official journal of the Controlled Release Society. 2007;121(1-2):100-9. Epub 2007/07/03.

17. Ehrhardt C, Fiegel J, Fuchs S, Abu-Dahab R, Schaefer UF, Hanes J, et al. Drug absorption by the respiratory mucosa: cell culture models and particulate drug carriers. Journal of aerosol medicine : the official journal of the International Society for Aerosols in Medicine. 2002;15(2):131-9. Epub 2002/08/20.

18. Fiegel J, Ehrhardt C, Schaefer UF, Lehr CM, Hanes J. Large porous particle impingement on lung epithelial cell monolayers--toward improved particle characterization in the lung. Pharmaceutical research. 2003;20(5):788-96. Epub 2003/05/20.

19. Fu J, Fiegel J, Krauland E, Hanes J. New polymeric carriers for controlled drug delivery following inhalation or injection. Biomaterials. 2002;23(22):4425-33. Epub 2002/09/11.

20. Gupta V, Ahsan F. Influence of PEI as a core modifying agent on PLGA microspheres of PGE(1), a pulmonary selective vasodilator. International journal of pharmaceutics. 2011;413(1-2):51-62. Epub 2011/05/03.

21. Gupta V, Davis M, Hope-Weeks LJ, Ahsan F. PLGA microparticles encapsulating prostaglandin E1-hydroxypropyl-beta-cyclodextrin (PGE1-HPbetaCD) complex for the treatment of pulmonary arterial hypertension (PAH). Pharmaceutical research. 2011;28(7):1733-49. Epub 2011/06/01.

22. Kim I, Byeon HJ, Kim TH, Lee ES, Oh KT, Shin BS, et al. Doxorubicin-loaded highly porous large PLGA microparticles as a sustained- release inhalation system for the

treatment of metastatic lung cancer. Biomaterials. 2012;33(22):5574-83. Epub 2012/05/15.

23. Meenach SA, Kim YJ, Kauffman KJ, Kanthamneni N, Bachelder EM, Ainslie KM. Synthesis, optimization, and characterization of camptothecin-loaded acetalated dextran porous microparticles for pulmonary delivery. Molecular pharmaceutics. 2012;9(2):290-8. Epub 2011/12/14.

24. Ungaro F, De Rosa G, Miro A, Quaglia F, La Rotonda MI. Cyclodextrins in the production of large porous particles: development of dry powders for the sustained release of insulin to the lungs. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2006;28(5):423-32. Epub 2006/06/30.

25. Yang Y, Bajaj N, Xu P, Ohn K, Tsifansky MD, Yeo Y. Development of highly porous large PLGA microparticles for pulmonary drug delivery. Biomaterials. 2009;30(10):1947-53. Epub 2009/01/13.

26. Ganji F, Vasheghani-Farahani E. Hydrogels in Controlled Drug Delivery Systems. Iranian Polymer Journal. 2008;18(1):63-88.

27. Beck-Broichsitter M, Rieger M, Reul R, Gessler T, Seeger W, Schmehl T. Correlation of drug release with pulmonary drug absorption profiles for nebulizable liposomal formulations. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2012. Epub 2012/12/25.

28. Biswas S, Deshpande PP, Perche F, Dodwadkar NS, Sane SD, Torchilin VP. Octa-arginine-modified pegylated liposomal doxorubicin: An effective treatment strategy for non-small cell lung cancer. Cancer letters. 2013. Epub 2013/02/20.

29. Monforte V, Lopez-Sanchez A, Zurbano F, Ussetti P, Sole A, Casals C, et al. Prophylaxis with nebulized liposomal amphotericin B for Aspergillus infection in lung transplant patients does not cause changes in the lipid content of pulmonary surfactant. The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation. 2013;32(3):313-9. Epub 2013/01/22.

30. Murata M, Yonamine T, Tanaka S, Tahara K, Tozuka Y, Takeuchi H. Surface modification of liposomes using polymer-wheat germ agglutinin conjugates to improve the absorption of peptide drugs by pulmonary administration. Journal of pharmaceutical sciences. 2013;102(4):1281-9. Epub 2013/02/08.

31. Seguin J, Brulle L, Boyer R, Lu YM, Ramos Romano M, Touil YS, et al. Liposomal encapsulation of the natural flavonoid fisetin improves bioavailability and antitumor efficacy. International journal of pharmaceutics. 2013;444(1-2):146-54. Epub 2013/02/06.

32. Zhou J, Zhao WY, Ma X, Ju RJ, Li XY, Li N, et al. The anticancer efficacy of paclitaxel liposomes modified with mitochondrial targeting conjugate in resistant lung cancer. Biomaterials. 2013;34(14):3626-38. Epub 2013/02/21.

33. Joshi MR, Misra AN. Liposomes of terbutaline sulphate: in vitro and in vivo studies. Indian journal of experimental biology. 1999;37(9):881-7. Epub 2000/02/25.

34. Chougule M, Padhi B, Misra A. Nano-liposomal dry powder inhaler of tacrolimus: preparation, characterization, and pulmonary pharmacokinetics. Int J Nanomedicine. 2007;2(4):675-88.

35. Thomas DA, Myers MA, Wichert B, Schreier H, Gonzalez-Rothi RJ. Acute effects of liposome aerosol inhalation on pulmonary function in healthy human volunteers. Chest. 1991;99(5):1268-70. Epub 1991/05/01.

36. Nguyen XC, Herberger JD, Burke PA. Protein powders for encapsulation: a comparison of spray-freeze drying and spray drying of darbepoetin alfa. Pharmaceutical research. 2004;21(3):507-14. Epub 2004/04/09.

37. Fiegel J, Fu J, Hanes J. Poly(ether-anhydride) dry powder aerosols for sustained drug delivery in the lungs. Journal of controlled release : official journal of the Controlled Release Society. 2004;96(3):411-23. Epub 2004/05/04.

38. Craig Dunbara, Gerhard Scheuchb, Knut Sommererb, Mark DeLonga, Alka Vermac, Batyckya R. In vitro and in vivo dose delivery characteristics of large porous particles for inhalation. International journal of pharmaceutics. 2002;245(1-2):179-89.

39. Oh YJ, Lee J, Seo JY, Rhim T, Kim SH, Yoon HJ, et al. Preparation of budesonide-loaded porous PLGA microparticles and their therapeutic efficacy in a murine asthma model. Journal of controlled release : official journal of the Controlled Release Society. 2011;150(1):56-62. Epub 2010/11/13.

40. Sivadas N, Cryan SA. Inhalable, bioresponsive microparticles for targeted drug delivery in the lungs. The Journal of pharmacy and pharmacology. 2011;63(3):369-75. Epub 2011/07/14.

41. Upadhyay D, Scalia S, Vogel R, Wheate N, Salama RO, Young PM, et al. Magnetised thermo responsive lipid vehicles for targeted and controlled lung drug delivery. Pharmaceutical research. 2012;29(9):2456-67. Epub 2012/05/16.

42. Hoare T, Kohane D. Hydrogels in drug delivery: Progress and challenges. Polymer. 2008;49:1993-2007.

43. Gonzalez-Alvarez M, Gonzalez-Alvarez I, Bermejo M. Hydrogels: an interesting strategy for smart drug delivery. Therapeutic delivery. 2013;4(2):157-60. Epub 2013/01/25.

44. Adi H, Young PM, Chan HK, Salama R, Traini D. Controlled release antibiotics for dry powder lung delivery. Drug development and industrial pharmacy. 2010;36(1):119-26. Epub 2009/08/07.

45. Kim B, Lim SH, Ryoo W. Preparation and characterization of pH-sensitive anionic hydrogel microparticles for oral protein-delivery applications. Journal of biomaterials science Polymer edition. 2009;20(4):427-36. Epub 2009/02/21.

46. Ruff LE, Mahmoud EA, Sankaranarayanan J, Morachis JM, Katayama CD, Corr M, et al. Antigen-loaded pH-sensitive hydrogel microparticles are taken up by dendritic cells with no requirement for targeting antibodies. Integrative biology : quantitative biosciences from nano to macro. 2013;5(1):195-203. Epub 2012/10/13.

47. Huynh CT, Nguyen MK, Jeong IK, Kim SW, Lee DS. Synthesis, Characteristics and Potential Application of Poly(beta-Amino Ester Urethane)-Based Multiblock Co-Polymers as an Injectable, Biodegradable and pH/Temperature-Sensitive Hydrogel System. Journal of biomaterials science Polymer edition. 2011. Epub 2011/05/31.

48. Niranjan R, Koushik C, Saravanan S, Moorthi A, Vairamani M, Selvamurugan N. A novel injectable temperature-sensitive zinc doped chitosan/beta-glycerophosphate hydrogel for bone tissue engineering. International journal of biological macromolecules. 2013;54:24-9. Epub 2012/12/04.

49. Niamlang S, Sirivat A. Electric field assisted transdermal drug delivery from salicylic acid-loaded polyacrylamide hydrogels. Drug delivery. 2009;16(7):378-88. Epub 2009/07/25.

50. Namdeo M, Bajpai SK, Kakkar S. Preparation of a magnetic-field-sensitive hydrogel and preliminary study of its drug release behavior. Journal of biomaterials science Polymer edition. 2009;20(12):1747-61. Epub 2009/09/03.

51. Perez RA, Won JE, Knowles JC, Kim HW. Naturally and synthetic smart composite biomaterials for tissue regeneration. Advanced drug delivery reviews. 2012. Epub 2012/04/03.

52. Satarkar NS, Hilt JZ. Magnetic hydrogel nanocomposites for remote controlled pulsatile drug release. Journal of controlled release : official journal of the Controlled Release Society. 2008;130(3):246-51. Epub 2008/07/09.

53. Singh S, Topuz F, Hahn K, Albrecht K, Groll J. Embedding of Active Proteins and Living Cells in Redox-Sensitive Hydrogels and Nanogels through Enzymatic Cross-Linking. Angewandte Chemie (International ed in English). 2013;52(10):3000-3. Epub 2013/02/07.

54. Vemula PK, Wiradharma N, Ankrum JA, Miranda OR, John G, Karp JM. Prodrugs as self-assembled hydrogels: a new paradigm for biomaterials. Current opinion in biotechnology. 2013. Epub 2013/03/08.

55. Kushwaha SK, Saxena P, Rai A. Stimuli sensitive hydrogels for ophthalmic drug delivery: A review. International journal of pharmaceutical investigation. 2012;2(2):54-60. Epub 2012/11/03.

56. Yoncheva K, Doytchinova I, Tankova L. Preparation and evaluation of isosorbide mononitrate hydrogels for topical fissure treatment. Current drug delivery. 2012;9(5):452-8. Epub 2010/02/18.

57. Beck-Broichsitter M, Schweiger C, Schmehl T, Gessler T, Seeger W, Kissel T. Characterization of novel spray-dried polymeric particles for controlled pulmonary drug delivery. Journal of controlled release : official journal of the Controlled Release Society. 2012;158(2):329-35. Epub 2011/11/09.

58. Ong HX, Traini D, Bebawy M, Young PM. Epithelial profiling of antibiotic controlled release respiratory formulations. Pharmaceutical research. 2011;28(9):2327-38. Epub 2011/05/27.

59. Wanakule P, Liu GW, Fleury AT, Roy K. Nano-inside-micro: Disease-responsive microgels with encapsulated nanoparticles for intracellular drug delivery to the deep lung. Journal of controlled release : official journal of the Controlled Release Society. 2012;162(2):429-37. Epub 2012/07/31.

60. Selvam P, El-Sherbiny IM, Smyth HD. Swellable hydrogel particles for controlled release pulmonary administration using propellant-driven metered dose

inhalers. Journal of aerosol medicine and pulmonary drug delivery. 2011;24(1):25-34. Epub 2010/10/22.

61. El-Sherbiny IM, Smyth HD. Biodegradable nano-micro carrier systems for sustained pulmonary drug delivery: (I) self-assembled nanoparticles encapsulated in respirable/swellable semi-IPN microspheres. International journal of pharmaceutics. 2010;395(1-2):132-41. Epub 2010/06/29.

62. El-Sherbiny I, Smyth H. Smart Magnetically Responsive Hydrogel Nanoparticles Prepared by a Novel Aerosol-Assisted Method for Biomedical and Drug Delivery Applications. Journal of Nanomaterials. 2011;2011.

63. Rytting E, Nguyen J, Wang X, Kissel T. Biodegradable polymeric nanocarriers for pulmonary drug delivery. Expert opinion on drug delivery. 2008;5(6):629-39. Epub 2008/06/06.

64. Steimer A, Haltner E, Lehr CM. Cell culture models of the respiratory tract relevant to pulmonary drug delivery. Journal of aerosol medicine : the official journal of the International Society for Aerosols in Medicine. 2005;18(2):137-82. Epub 2005/06/22.

65. Houtmeyers E, Gosselink R, Gayan-Ramirez G, Decramer M. Regulation of mucociliary clearance in health and disease. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 1999;13(5):1177-88. Epub 1999/07/22.

66. fiegel J, Brenza T, Hamed R. Controlled Treansport for pulmonary Drug Delivery. Smyth HDC, Hickey AJ, editors. New York: Springer 2011.

67. Geiser M. Update on macrophage clearance of inhaled micro- and nanoparticles. Journal of aerosol medicine and pulmonary drug delivery. 2010;23(4):207-17. Epub 2010/01/30.

68. He C, Hu Y, Yin L, Tang C, Yin C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. Biomaterials. 2010;31(13):3657-66. Epub 2010/02/09.

69. Mahmud A, Discher DE. Lung vascular targeting through inhalation delivery: insight from filamentous viruses and other shapes. IUBMB life. 2011;63(8):607-12. Epub 2011/07/02.

70. Balasubramanian SK, Poh KW, Ong CN, Kreyling WG, Ong WY, Yu LE. The effect of primary particle size on biodistribution of inhaled gold nano-agglomerates. Biomaterials. 2013;34(22):5439-52. Epub 2013/05/04.

71. Gehr P, Geiser M, Hof V, Schürch S, Waber U, Baumann M. Surfactant and inhaled particles in the conducting airways: Structural, stereological, and biophysical aspects. Microscopy research technique. 1993;26(5):423-36.

72. Edwards DA, Hanes J, Caponetti G, Hrkach J, Ben-Jebria A, Eskew ML, et al. Large porous particles for pulmonary drug delivery. Science (New York, NY). 1997;276(5320):1868-71. Epub 1997/06/20.

73. Sharma G, Mueannoom W, Buanz AB, Taylor KM, Gaisford S. In vitro characterisation of terbutaline sulphate particles prepared by thermal ink-jet spray freeze drying. International journal of pharmaceutics. 2013. Epub 2013/03/05.

74. Watts AB, Wang YB, Johnston KP, Williams RO, 3rd. Respirable low-density microparticles formed in situ from aerosolized brittle matrices. Pharmaceutical research. 2013;30(3):813-25. Epub 2012/12/12.

75. Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, et al. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. Environmental health perspectives. 2005;113(11):1555-60. Epub 2005/11/03.

76. Todoroff J, Vanbever R. fate of nanomedicine in the lungs. Current Opinion in Colloid & Interface Science. 2011;16(3):246-54.

77. Beck-Broichsitter M, Gauss J, Packhaeuser CB, Lahnstein K, Schmehl T, Seeger W, et al. Pulmonary drug delivery with aerosolizable nanoparticles in an ex vivo lung model. International journal of pharmaceutics. 2009;367(1-2):169-78. Epub 2008/10/14.

78. Grenha A, Seijo B, Remunan-Lopez C. Microencapsulated chitosan nanoparticles for lung protein delivery. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2005;25(4-5):427-37. Epub 2005/05/17.

79. Sham JO, Zhang Y, Finlay WH, Roa WH, Lobenberg R. Formulation and characterization of spray-dried powders containing nanoparticles for aerosol delivery to the lung. International journal of pharmaceutics. 2004;269(2):457-67. Epub 2004/01/07.

80. Yang W, Peters JI, Williams RO, 3rd. Inhaled nanoparticles--a current review. International journal of pharmaceutics. 2008;356(1-2):239-47. Epub 2008/03/25.

81. Creutzenberg O, Bellmann B, Korolewitz R, Koch W, Mangelsdorf I, Tillmann T, et al. Change in agglomeration status and toxicokinetic fate of various nanoparticles in vivo following lung exposure in rats. Inhalation toxicology. 2012;24(12):821-30. Epub 2012/10/05.

82. Morfeld P, Treumann S, Ma-Hock L, Bruch J, Landsiedel R. Deposition behavior of inhaled nanostructured TiO2 in rats: fractions of particle diameter below 100 nm (nanoscale) and the slicing bias of transmission electron microscopy. Inhalation toxicology. 2012;24(14):939-51. Epub 2012/12/12.

83. El-Sherbiny IM, McGill S, Smyth HD. Swellable microparticles as carriers for sustained pulmonary drug delivery. Journal of pharmaceutical sciences. 2010;99(5):2343-56. Epub 2009/12/08.

84. El-Sherbiny IM, Smyth HD. Poly(ethylene glycol)-carboxymethyl chitosan-based pH-responsive hydrogels: photo-induced synthesis, characterization, swelling, and in vitro evaluation as potential drug carriers. Carbohydrate research. 2010;345(14):2004-12. Epub 2010/08/17.

85. Sakagami M, Kinoshita W, Sakon K, Sato J, Makino Y. Mucoadhesive beclomethasone microspheres for powder inhalation: their pharmacokinetics and pharmacodynamics evaluation. Journal of controlled release : official journal of the Controlled Release Society. 2002;80(1-3):207-18. Epub 2002/04/12.

86. Surendrakumar K, Martyn GP, Hodgers EC, Jansen M, Blair JA. Sustained release of insulin from sodium hyaluronate based dry powder formulations after

pulmonary delivery to beagle dogs. Journal of controlled release : official journal of the Controlled Release Society. 2003;91(3):385-94. Epub 2003/08/23.

87. Hwang SM, Kim DD, Chung SJ, Shim CK. Delivery of ofloxacin to the lung and alveolar macrophages via hyaluronan microspheres for the treatment of tuberculosis. Journal of controlled release : official journal of the Controlled Release Society. 2008;129(2):100-6. Epub 2008/06/10.

88. Lo YL, Hsu CY, Lin HR. pH-and thermo-sensitive pluronic/poly(acrylic acid) in situ hydrogels for sustained release of an anticancer drug. Journal of drug targeting. 2013;21(1):54-66. Epub 2012/09/27.

89. Luppi B, Bigucci F, Cerchiara T, Zecchi V. Chitosan-based hydrogels for nasal drug delivery: from inserts to nanoparticles. Expert opinion on drug delivery. 2010;7(7):811-28. Epub 2010/06/22.

90. Akala EO, Kopeckova P, Kopecek J. Novel pH-sensitive hydrogels with adjustable swelling kinetics. Biomaterials. 1998;19(11-12):1037-47. Epub 1998/08/06.

91. Chen K, Zhang Q, Chen L. Effect of Cross-Linking Degree on Hydrogels Using Surfactant Detergent as Template. Adanced Materials Research 2011;284-286:1827-30.

92. Guvendiren M, Burdick J, Yang S. Kinetic study of swelling-induced surface pattern formation and ordering in hydrogel films with depth-wise crosslinking gradient. Soft matter. 2010;6(9):2044-9.

93. Hennink WE, van Nostrum CF. Novel crosslinking methods to design hydrogels. Advanced drug delivery reviews. 2002;54(1):13-36. Epub 2002/01/05.

94. Boura C, Menu P, Payan E, Picart C, Voegel JC, Muller S, et al. Endothelial cells grown on thin polyelectrolyte mutlilayered films: an evaluation of a new versatile surface modification. Biomaterials. 2003;24(20):3521-30. Epub 2003/06/18.

95. Boura C, Muller S, Vautier D, Dumas D, Schaaf P, Claude Voegel J, et al. Endothelial cell--interactions with polyelectrolyte multilayer films. Biomaterials. 2005;26(22):4568-75. Epub 2005/02/22.

96. Schneider GB, English A, Abraham M, Zaharias R, Stanford C, Keller J. The effect of hydrogel charge density on cell attachment. Biomaterials. 2004;25(15):3023-8. Epub 2004/02/18.

97. Kim S, English AE, Kihm KD. Surface elasticity and charge concentration-dependent endothelial cell attachment to copolymer polyelectrolyte hydrogel. Acta biomaterialia. 2009;5(1):144-51. Epub 2008/09/09.

98. Liu Y, Ibricevic A, Cohen JA, Cohen JL, Gunsten SP, Frechet JM, et al. Impact of hydrogel nanoparticle size and functionalization on in vivo behavior for lung imaging and therapeutics. Molecular pharmaceutics. 2009;6(6):1891-902. Epub 2009/10/27.

99. Mobus K, Siepmann J, Bodmeier R. Zinc-alginate microparticles for controlled pulmonary delivery of proteins prepared by spray-drying. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2012;81(1):121-30. Epub 2012/02/22.

100. Hezaveh H, Muhamad, II. Impact of metal oxide nanoparticles on oral release properties of pH-sensitive hydrogel nanocomposites. International journal of biological macromolecules. 2012;50(5):1334-40. Epub 2012/04/10.

101. Mihaila SM, Gaharwar AK, Reis RL, Marques AP, Gomes ME, Khademhosseini A. Photocrosslinkable Kappa-Carrageenan Hydrogels for Tissue Engineering Applications. Advanced healthcare materials. 2012. Epub 2013/01/03.

102. Popa EG, Caridade SG, Mano JF, Reis RL, Gomes ME. Chondrogenic potential of injectable kappa-carrageenan hydrogel with encapsulated adipose stem cells for cartilage tissue-engineering applications. Journal of tissue engineering and regenerative medicine. 2013. Epub 2013/01/11.

103. Popa EG, Gomes ME, Reis RL. Cell delivery systems using alginate--carrageenan hydrogel beads and fibers for regenerative medicine applications. Biomacromolecules. 2011;12(11):3952-61. Epub 2011/10/06.

104. Salgueiro AM, Daniel-da-Silva AL, Fateixa S, Trindade T. kappa-Carrageenan hydrogel nanocomposites with release behavior mediated by morphological distinct Au nanofillers. Carbohydrate polymers. 2013;91(1):100-9. Epub 2012/10/10.

105. El-Menshawe SF, Hussein AK. Formulation and evaluation of meloxicam niosomes as vesicular carriers for enhanced skin delivery. Pharmaceutical development and technology. 2011. Epub 2011/09/15.

106. Hezaveh H, Muhamad, II, Noshadi I, Shu Fen L, Ngadi N. Swelling behaviour and controlled drug release from cross-linked kappa-carrageenan/NaCMC hydrogel by diffusion mechanism. Journal of microencapsulation. 2012;29(4):368-79. Epub 2012/02/09.

107. Juntapram K, Praphairaksit N, Siraleartmukul K, Muangsin N. Electrosprayed polyelectrolyte complexes between mucoadhesive N,N,N,-trimethylchitosan-

homocysteine thiolactone and alginate/carrageenan for camptothecin delivery. Carbohydrate polymers. 2012;90(4):1469-79. Epub 2012/09/05.

108. Kulkarni RV, Boppana R, Krishna Mohan G, Mutalik S, Kalyane NV. pH-responsive interpenetrating network hydrogel beads of poly(acrylamide)-g-carrageenan and sodium alginate for intestinal targeted drug delivery: synthesis, in vitro and in vivo evaluation. Journal of colloid and interface science. 2012;367(1):509-17. Epub 2011/11/04.

109. Yamada K, Kamada N, Odomi M, Okada N, Nabe T, Fujita T, et al. Carrageenans can regulate the pulmonary absorption of antiasthmatic drugs and their retention in the rat lung tissues without any membrane damage. International journal of pharmaceutics. 2005;293(1-2):63-72. Epub 2005/03/22.

110. Fan H, Dash AK. Effect of cross-linking on the in vitro release kinetics of doxorubicin from gelatin implants. International journal of pharmaceutics. 2001;213(1-2):103-16. Epub 2001/02/13.

111. Podczeck F, Jones B. Pharmaceutical Capsules, 2nd edn.: Pharmaceutical Press; 2004.

112. Tu J, Wang L, Yang J, Fei H, Li X. Formulation and pharmacokinetic studies of acyclovir controlled-release capsules. Drug development and industrial pharmacy. 2001;27(7):687-92. Epub 2001/11/06.

113. Rouse JJ, Whateley TL, Thomas M, Eccleston GM. Controlled drug delivery to the lung: Influence of hyaluronic acid solution conformation on its adsorption to

hydrophobic drug particles. International journal of pharmaceutics. 2007;330(1-2):175-82. Epub 2007/01/09.

114. Baier Leach J, Bivens KA, Patrick CW, Jr., Schmidt CE. Photocrosslinked hyaluronic acid hydrogels: natural, biodegradable tissue engineering scaffolds. Biotechnology and bioengineering. 2003;82(5):578-89. Epub 2003/03/26.

115. Park YD, Tirelli N, Hubbell JA. Photopolymerized hyaluronic acid-based hydrogels and interpenetrating networks. Biomaterials. 2003;24(6):893-900. Epub 2002/12/31.

116. Shu Z, Liu X, Palumbo Y, Luo FS, Prestwich Y. In situ crosslinkable hyaluronan hydrogels for tissue engineering. Biomaterials. 2004;25(7-8):1339-48. Epub 2003/12/04.

117. Yeo Y, Highley CB, Bellas E, Ito T, Marini R, Langer R, et al. In situ cross-linkable hyaluronic acid hydrogels prevent post-operative abdominal adhesions in a rabbit model. Biomaterials. 2006;27(27):4698-705. Epub 2006/06/06.

118. Morimoto K, Metsugi K, Katsumata H, Iwanaga K, Kakemi M. Effects of low-viscosity sodium hyaluronate preparation on the pulmonary absorption of rh-insulin in rats. Drug development and industrial pharmacy. 2001;27(4):365-71. Epub 2001/06/20.

119. Gojgini S, Tokatlian T, Segura T. Utilizing cell-matrix interactions to modulate gene transfer to stem cells inside hyaluronic acid hydrogels. Molecular pharmaceutics. 2011;8(5):1582-91. Epub 2011/08/10.

120. Segura T, Anderson BC, Chung PH, Webber RE, Shull KR, Shea LD. Crosslinked hyaluronic acid hydrogels: a strategy to functionalize and pattern. Biomaterials. 2005;26(4):359-71. Epub 2004/07/28.

121. Xu X, Jha AK, Harrington DA, Farach-Carson MC, Jia X. Hyaluronic Acid-Based Hydrogels: from a Natural Polysaccharide to Complex Networks. Soft matter. 2012;8(12):3280-94. Epub 2012/03/16.

122. Son YJ, McConville JT. Advancements in dry powder delivery to the lung. Drug development and industrial pharmacy. 2008;34(9):948-59. Epub 2008/09/19.

123. Ahsan F, Rivas IP, Khan MA, Torres Suarez AI. Targeting to macrophages: role of physicochemical properties of particulate carriers--liposomes and microspheres--on the phagocytosis by macrophages. Journal of controlled release : official journal of the Controlled Release Society. 2002;79(1-3):29-40. Epub 2002/02/21.

124. Champion JA, Walker A, Mitragotri S. Role of particle size in phagocytosis of polymeric microspheres. Pharmaceutical research. 2008;25(8):1815-21. Epub 2008/04/01.

125. Makino K, Yamamoto N, Higuchi K, Ohshima H, Terada H. Phagocytic uptake of polystyrene microspheres by alveolar macrophages: effects of the size and surface properties of the microspheres Colloids and Surfaces B: Biointerfaces. 2003;27:33-9.

126. El-Sherbiny IM, Smyth HD. Controlled release pulmonary administration of curcumin using swellable biocompatible microparticles. Molecular pharmaceutics. 2012;9(2):269-80. Epub 2011/12/06.

127. Garcia-Contreras L. In Vivo Animal Models for Controlled-Relase Pulmonary Drug Delivery. Smyth HDC, Hickey AJ, editors. New York: Springer 2011.

128. Rossi SE, Erasmus JJ, McAdams HP, Sporn TA, Goodman PC. Pulmonary drug toxicity: radiologic and pathologic manifestations. Radiographics : a review publication

of the Radiological Society of North America, Inc. 2000;20(5):1245-59. Epub 2000/09/19.

129. Singh G, Poochikian G. Development and Approval of Inhaled Respiratory Drugs: A US Regulatory Science Perspective. Smyth HDC, Hickey AJ, editors. New York: Springer 2011.

130. Vandevanter DR, Geller DE. Tobramycin administered by the TOBI((R)) Podhaler((R)) for persons with cystic fibrosis: a review. Medical devices (Auckland, NZ). 2011;4:179-88. Epub 2011/01/01.

Chapter 3: Research objective

Recently, the use of inhalation aerosols to treat the Cystic Fibrosis directly has improved treatment and reduced side effects. However, even the obvious advantages of aerosols over systemic therapy, current inhaled medications for Cystic Fibrosis are still inefficient, time consuming. Plus, most of Cystic Fibrosis patients exhibit reduced airway diameters due to inflammation, increased mucus production, frequently infection, and combinations of these states. Those properties of Cystic Fibrosis directly lead to the inefficient or even fail of most aerosolization. Therefore, the design of efficient drug delivery should consider the physiological changes, such as airway obstruction, kinetics of drug diffusion through mucus, drug release and excipients degradation in the inflammatory environment. The objective of this proposal is to evaluate the delivery properties of aerosol particles which are easily formed, to overcome the physiological barriers in Cystic Fibrosis.

Our first hypothesis is that ciprofloxacin can play a role of cross-linker, similar with Calcium, to form hydrogel with alginate which is a natural biodegradable polymer. Furthermore, this novel and easily formed hydrogel can release the drug in a sustained release profile, which is preferred in treatment of Cystic Fibrosis. In the proposal study, we will utilize the antibiotics, ciprofloxacin to cross-link the alginate to finally form a hydrogel, which also entraps free ciprofloxacin. We also will evaluate its aerodynamic performance suitable for aerosolization. Through cell based assay, to test its swelling properties, which allow delayed macrophage uptake. Moreover, we will evaluate this swellable hydrogel in an animal model to demonstrate its sustained release property.

In a recent formulation investigated in our laboratory, a copolymer of PEG grafted onto phthaloyl chitosan (PEG-g-PHCs) self-assembled nanoparticles were entrapped into Ca^{2+} cross-linked alginate to prepare respirable microparticles with semi-interpenetrating polymer networks (semi-IPN particles) for pulmonary sustained drug delivery. In **Chapter 4**, a nano-in-micro hydrogel particle formulation was developed for sustained pulmonary drug delivery, which takes the advantages of both nanoparticles and swellable and respirable hydrogel particles. The dry nano-in-micro hydrogel particles exhibited a rapid initial swelling within 2 minutes, and showed sustained drug release. Preliminary in vivo pharmacokinetic studies were performed with formulations delivered to rats by intratracheal insufflation. Ciprofloxacin concentrations in plasma and in lung tissue and lavage were measured up to 7 hr. The swellable particles showed a lower ciprofloxacin levels in plasma than the controlled group (a mixture of lactose with micronized ciprofloxacin); while swellable particles achieved higher concentrations in lung tissue and lavage, indicating the swellable particles could be used for controlling drug release and prolonging lung drug concentrations

In the chapter 4, we had reported that ciprofloxacin can function as a cross-linker to interact with alginate to form hydrogel. Based on this interaction and the fact of the complex and costly process of making PEGylated chitosan, we therefore directly combined ciprofloxacin with alginate to form hydrogel dry powder, without the addition of extra chitosan in **Chapter 5**. In such way, we simplified the preparation method for hydrogel particles, decreased the potential risk of polymeric chitosan to the lung tissue, and increased the ciprofloxacin loading efficiency from 30% to 50% in the finial microsized hydrogel dry powder. The alginate hydrogel dry powder system exhibited

high ciprofloxacin loading (57%) and a geometric size of less than 5 µm. Ciprofloxacin was present in the amorphous state in the dry powder and was released in a controlled release manner relative to ciprofloxacin alone, i.e. 80% of drug released at 8 hours. The hydrogel dry powder also achieved a high fine particle fraction (above 45%) as determined by the *in vitro* aerosol performance study. A novel inhalable alginate hydrogel dry powder system was successfully formed and it indicated broader applications of other antibiotics for the treatment of lung infections.

Our second hypothesis, derived from the proposed mechanisms of biofilm resistances, is that reducing the binding or affinity of the antibacterial toward the biofilm itself will result in improved antibiotic efficacy. To test this hypothesis, in **Chapter 6**, a conventional antibiotic, tobramycin was chemically modified. Tobramycin has previously been demonstrated to bind to biofilm matrices, thus reducing the effective concentration of antimicrobial able to reach the pathogenic organisms, as well as limiting the penetration of the antibacterial agent to the deeper microstructure of the biofilm, thereby creating an undesirable stress response in the pathogen. It is essential to improve the penetrative capabilities of existing antimicrobials, such as tobramycin, in order to overcome thick biofilm barriers and to achieve superior elimination of P. aeruginosa biofilms. Modifying existing drugs by conjugating them to polymers has been widely reported to improve the efficacy of existing drugs. Predominantly, conjugation to polyethylene glycol (PEG) has been used to increase plasma half-lives of therapeutic agents. PEGylation has also been shown to improve diffusion of nanoparticles through mucus. To our knowledge, the conjugation of PEG to tobramycin has not been reported in the literature, but its feasibility is supported by reports that have shown that chemical

modification at the 6’ amine group of tobramycin will still maintain antibacterial activity. The minimum inhibitory concentration (MIC_{80}) of Tob-PEG was higher (13.9 µmol/L) than that of tobramycin (1.4 µmol/L) in the planktonic phases. In contrast, the Tob-PEG was approximately 3.2 fold more effective in eliminating bacterial biofilms than tobramycin. Specifically, Tob-PEG had MIC80 lower than those exhibited by tobramycin (27.8 µmol/L vs 89.8 µmol/L). Confocal laser scanning microscope and scanning electron microscope findings further confirmed these data. Thus, modification of antimicrobial as by PEGylation appears to be a promising approach for overcoming the bacterial resistance in the established biofilms of Pseudomonas aeruginosa.

Chapter 4: Swellable ciprofloxacin-loaded nano-in-micro hydrogel particles for local lung drug delivery [1, 2]

4.1 ABSTRACT

Incorporation of drug-loaded nanoparticles into swellable and respirable micro particles is a promising strategy to avoid rapid clearance from the lung and achieve sustained drug release. In this investigation, a co-polymer of Polyethylene glycol grafted onto phthaloyl chitosan (PEG-g-PHCs) was synthesized, and then self-assembled with ciprofloxacin to form drug-loaded nanoparticles. The nanoparticles and free drug were encapsulated into respirable and swellable alginate micro hydrogel particles and assessed as a novel system for pulmonary sustained drug delivery. Particle size, morphology, dynamic swelling profile and *in vitro* drug release were investigated. Results showed that drug-loaded nanoparticles with size of 218 nm were entrapped into 3.9 μm micro hydrogel particles. The dry nano-in-micro hydrogel particles exhibited a rapid initial swelling within 2 minutes, and showed sustained drug release. Preliminary *in vivo* pharmacokinetic studies were performed with formulations delivered to rats by intratracheal insufflation. Ciprofloxacin concentrations in plasma and in lung tissue and lavage were measured up to 7 hr.

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2. Statement of co-author contribution. This chapter was written by Ju Du; some sections were written by Dr. Ibrahim M. El-Sherbiny. Dr. Hugh Smyth helped with editorial and content assistance. The *in vitro* methods and results in this chapter were made by Dr. Ibrahim M. El-Sherbiny, and the *in vivo* methods and results were made by Ju Du.

The swellable particles showed a lower ciprofloxacin levels in plasma than the controlled group (a mixture of lactose with micronized ciprofloxacin); while swellable particles achieved higher concentrations in lung tissue and lavage, indicating the swellable particles could be used for controlling drug release and prolonging lung drug concentration.

4.2 INTRODUCTION

Delivering antibiotics through the pulmonary route increases the local drug concentration in the lung, leading to improved local antibacterial effect in lung infections (1, 2). In patients with lung infections, such as cystic fibrosis and pneumonia, the reduction of administration frequency (3), dose (4) and duration of inhalation treatment (5) will increase the patient's compliance and adherence to the therapy (6). The administration frequency of inhalation aerosols could be reduced by prolonging the residence time of drug-releasing particles in the lung, essentially by using a sustained release formulation (7). However, there are few effective sustained release pulmonary formulations developed currently because of the efficient clearance of inhaled particles either by mucociliary escalator (8) or alveolar macrophage uptake (9).

For effective delivery of sustained release formulations into the lung, drug-loaded particles should have suitable aerodynamic properties, which is primarily determined either by the particle size, shape and particle density. The preferred aerodynamic particle size range for deep lung delivery is around 0.5-5μm (9). However, the conundrum is that particles within this size range are subject to rapid phagocytosis and are cleared by alveolar macrophages (10, 11). To overcome the clearance due to alveolar macrophage uptake, two main approaches, larger particles with low density (12) and nanoparticles (13, 14), have been explored. Low density particles with larger micro-size (i.e. greater than around 6 microns) have been shown to reduce the uptake rate by macrophages. However, there may be limitations of controlling the drug release from this type of particle system (15). Even though nanoparticles have shown promise in evading phagocytosis and mucociliary clearance, they may be exhaled easily following inhalation

resulting in lower deposition in the airways (16, 17). Additionally, it is critical that nanoparticles must not tend to aggregate together, forming microparticles which once again lead to rapid clearance by macrophages (15, 18).

Thus, it would be extremely useful to develop an alternative pulmonary delivery vector for increasing the residence time of the drugs in the lung. Such a delivery vector should have efficient deposition in the deep lung and diminish alveolar macrophage uptake. Acknowledging the physiological barriers to successful pulmonary drug delivery of controlled release systems, our group has worked on a third approach for improving prolonged drug release in the lung. Specifically we have developed a particle platform around swellable hydrogel particles intended for inhalation (9, 19-21). The swellable particles have respirable aerodynamic size in dry state (i.e. during administration) but swell to larger geometric sizes after deposition in the hydrated wet respiratory tract, thus evading the macrophage uptake. Various cargos have been incorporated into these swellable particles, including the encapsulation of nanoparticles permitting controlled drug release (22).

In a recent formulation investigated in our laboratory, a copolymer of PEG grafted onto phthaloyl chitosan (PEG-g-PHCs) self-assembled nanoparticles were entrapped into Ca^{2+} cross-linked alginate to prepare respirable microparticles with semi-interpenetrating polymer networks (semi-IPN particles) for pulmonary sustained drug delivery. The preliminary *in vitro* evaluation of this hydrogel showed that it could be used as good potential carrier for pulmonary sustained drug delivery (15). Alginate is a natural, low toxicity, and biocompatible polyanionic polymer, composing of mannuronic acid (M) and guluronic acid (G) residues arranged linearly as consecutive G blocks (GGGGG), or

consecutive M blocks (MMMMM), or even alternating G and M blocks (GMGMGM) (23). The anionic alginate interacts with cationic chitosan (24), which is biocompatible and biodegradable polysaccharide (25) and is able to prolong drug release (26). In biomedical applications, alginate has been extensively investigated due to its unique ability to form hydrogel via ionotropic cross-linking with divalent cations such as Ca^{2+} (27, 28). However, it has been recently reported that calcium in alginate hydrogel stimulates an inflammatory response (29), which is undesirable in lungs for most therapeutic applications.

For biomedical applications of alginate, there has been debate about the immunogenicity of the polymer despite the extensive evaluations both *in vitro* and *in vivo* (23, 30). One contention related to alginate immunogenicity is associated with the ratio of alginate blocks (31, 32). Alginate consists of both M-block and G-block. It was reported that alginate with a high ratio of M-block to G-block was more immunogenic and thus triggered an increased release of cytokines than that with high ratio of G-block to M-block (33). In contrast, it has also been reported that no immunogenicity was observed when alginate with varied levels of M-block was investigated (34). Alginate immunogenicity may also be related to the impurities in different sources or batches of alginate used. Alginate is extracted from natural resources. Thus impurities such as heavy metals, endotoxins, proteins and polyphenolic compounds may be present in alginate that could potentially cause an immune response (23). But, no foreign body reaction was observed when alginate was processed via multi-step purification technique in an animal model (30, 35). Therefore, based on the literatures, it appears that in some cases alginate

polymer may exert immunogenic responses, but in others, alginate appears to be non-immunogenic.

In this manuscript, a nano-in-micro hydrogel particle formulation was developed for sustained pulmonary drug delivery, which takes the advantages of both nanoparticles and swellable and respirable hydrogel particles. Specifically we have developed an alginate hydrogel which was free of Ca^{2+}. PEG-g-PHCs was synthesized and then self-assembled into nanoparticles in combination with ciprofloxacin. The nanoparticle suspension was mixed with sodium alginate solution to form microparticle. In this microparticle, ciprofloxacin which was not incorporated into nanoparticles in the beginning would act as a cross-linker, similar to and replacing Ca^{2+}, to form hydrogel with alginate. Subsequently, the hydrogel particles were spray dried to dry swellable nano-in-micro hydrogel particles, which were further evaluated with *in vitro* and *in vivo* experiments in rats by intratracheal insufflation.

4.3 MATERIALS AND METHODS

4.3.1 Materials

4.3.1.1 Formulation study

Chitosan (Cs) (MW: 4-5×10^5 Da. %N-deacetylation: about 76.4 %), monomethoxy-poly(ethylene glycol) (m-PEG, Mn 5,000 Da), succinic anhydride and 1-hydroxybenzotrizole (HOBt) were obtained from Aldrich (Saint Louis, MO). 4-Dimethylaminopyridine (DMAP) and ciprofloxacin were purchased from Sigma (St Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCL) was provided by Fluka Chemical Corp. (Milwaukee, WI). Sodium alginate

(low viscosity; 250 cps for a 2% solution at 25^{o}C), triethyl amine and other chemicals were obtained from Sigma-Aldrich (St Louis, MO). Phosphate buffer saline (PBS, pH 7.4), absolute ethanol and all other reagents were of analytical grade and used as received.

4.3.1.2 Cell culture

Mouse macrophages cells, RAW 264.7 (8.8×10^{5} cells/mL) and Fetal bovine serum (FBS) were obtained from American Type Culture Collection, ATCC (Manassass, VA). Dulbecco's Modified Eagle Medium (DMEM) was provided by Gibco (Grand Island, NY). 1 µm polystyrene (PS) particles (Fluospheres fluorescent (505/515) and 112 µm PS particles were purchased from Invitrogen (Eugene, OR) and Bangs Laboratories, Inc.(Fishers, IN), respectively. Paraformaldehyde (PFA, 4%) solution was obtained from USB Corporation (Cleveland, Ohio).

4.3.1.3 Preliminary in vivo pharmacokinetic studies

Lactose (Respitose®ML001) was obtained from DMV-Fonterra Excipients. Methanol and acetonitrile purchased from Fisher Scientific. Insufflator was provided by Penn-Century DP-4, Penn Century (Philadelphia, PA).

4.3.2 Methods

4.3.2.1 Preparation of PEG graft copolymerized onto phthaloyl chitosan (PEG-g-PHCs)

The copolymer of PEG grafted onto phthaloyl chitosan (PHCs) was synthesized by a modified method reported in details in our earlier studies (9, 15), and described briefly as follows:

Firstly, PHCs was synthesized by reaction of 5 g of Cs with 22.5 g of phthalic anhydride in 150 mL of DMF at 130^{o}C under dry nitrogen atmosphere for 10 h. The reaction mixture was left to reach room temperature and then the PHCs was precipitated over ice-water. The precipitated PHCs was filtered, washed with ethanol, and freeze dried. Secondly, m-PEG was converted into m-PEG-COOH through reaction with succinic anhydride. In brief, 10 g of m-PEG, 0.24 g of DMAP, 0.24 g of succinic anhydride, and 0.2 g of triethylamine were dissolved in 50 mL of dry dioxane. The reaction mixture was stirred at room temperature for 2 days under dry nitrogen atmosphere. The dioxane was evaporated and the residue (m-PEG-COOH) was taken up in CCl_4, filtered and precipitated by diethyl ether. Lastly, the PEG-g-PHCs copolymer was obtained by stirring of 3.8 g of m-PEG-COOH with 0.5 g of the dried PHCs in 15 mL DMF. Then, 0.3 g of the HOBt was added and the reaction mixture was stirred at room temperature until obtaining a clear solution. Afterwards, EDC·HCl (0.43 g) was added and the reaction was continued overnight under stirring at room temperature. The obtained PEG-g-PHCs copolymer was purified by dialysis in distilled water, washed with ethanol and freeze dried.

4.3.2.2 Preparation of dry swellable nano-in-micro hydrogel particles

The Ciprofloxacin-loaded swellable hydrogel particles were obtained via spray drying of a combination of ciprofloxacin-loaded PEG-g-PHCs nanoparticles suspension and sodium alginate solution. Briefly, homogenous solutions of the ciprofloxacin-loaded PEG-g-PHCs nanoparticles (1% w/v) and sodium alginate (3% w/v) were prepared using 0.06% acetic acid and distilled water as solvents, respectively. The self-assembled PEG-g-PHCs nanoparticles were prepared by sonication of 1% w/v PEG-g-PHCs solution

containing ciprofloxacin using a probe type sonicator (Misonix ultrasonic processor, S-4000, Misonix Inc, CT) at 60 W for 2 minutes. The sonication step was repeated twice and performed in an ice-water bath. Then, a 75 mL of ciprofloxacin-loaded PEG-g-PHCs nanoparticles suspension was added dropwise with stirring to 25 mL of 3% aqueous alginate solution. The mixture was completed with distilled water up to a final concentration of 1.5% w/v. Then, the homogenized polymer mixture was spray-dried with a 0.7 mm two-fluid pressurized atomizer at a feed rate of 25% (6 mL/min) in a Büchi Mini spray dryer B-290 (Büchi, Switzerland). The atomizing air flow rate was 500-600 NL/h. The inlet temperature was adjusted at 125ºC and the outlet temperature varied between 60ºC and 65ºC. The obtained hydrogel particles powder was collected and the spray drying yield (%) was calculated.

4.3.2.3 Determination of particle size

The size of the ciprofloxacin-loaded PEG-g-PHCs nanoparticles was estimated using dynamic light scattering (Wyatt Technology Corporation Dyna Pro-titan DLS) after sonication of a 1% PEG-g-PHCs solution containing ciprofloxacin for 2 minutes at a power of 60 watt. Size of micro hydrogel particles was determined using laser diffraction (SYMPATEC, Sympatec Gmbt, System Partikl-Technik, Germany, He-Ne laser beam 5 mW max at 632.8 nm). The measurements were carried out in triplicates for the suspension of the micro hydrogel particles in acetone. Volume mean diameter (VMD, µm) was calculated from the particle size distribution curves for the micro hydrogel particles. Average aerodynamic diameter of the ciprofloxacin-loaded nano-in-micro hydrogel particles was also calculated using the following relationship (36):

$$daer = d\sqrt{\rho} \qquad (1)$$

Where, d_{aer} is the micro hydrogel particles aerodynamic diameter (µm); d is the geometric diameter (VMD, µm); ρ is the micro hydrogel particles tapped density (g/cc).

4.3.2.4 Morphology of micro hydrogel particles

The morphology of the prepared dry swellable ciprofloxacin-loaded nano-in-micro hydrogel particles was examined by scan electron microscope (SEM) (Hitachi S-800 field emission scanning electron microscope operated in secondary electron mode with a Robinson backscatter detector and with a Hitachi PCI system for digital image capture). Dry particles were mounted on aluminum stubs with double-sided conducting carbon tapes and coated with a 50/50 mixture of Au/Pd to minimize surface charging. The samples were scanned at an accelerating voltage of 20 KV.

4.3.2.5 Swelling study of micro hydrogel particles

The swelling pattern of the developed dry ciprofloxacin-loaded nano-in-micro hydrogel particles in PBS, pH 7.4, was studied by determining the increase in both VMD (µm) and the median diameter (X_{50}, µm) of the particles with time using laser diffractometer (SYMPATEC, Sympatec Gmbt, System Partikl-Technik, Germany).

4.3.2.6 Investigation of next generation impactor (NGI)

10 (±1) mg of powder, filled in size 3 Vcaps HPMC capsule, was dispersed through a commercial inhaler Handihaler® (Pfizer, Inc., USA; Boehringer Ingelheim, Inc. Germany) into a next generation impactor (NGI, MSP Corp, MN) at a volumetric flow rate of 60 $Lmin^{-1}$ actuated for 4-s. Drug content collected at each stage from the NGI apparatus was assessed via UV–VIS absorption spectroscopy at 280 nm. Emitted fraction (EF) was expressed as the total mass fraction of drug emitted from the inhaler. Fine

particle fraction (FPF) was defined as the drug mass (<5 μm) deposited in the NGI divided by the emitted dose. Respirable fraction (RF) was defined as the drug mass (<5 μm) deposited in the NGI divided by drug mass recovered from the entire system.

4.3.2.7 In vitro release of ciprofloxacin

The *in vitro* release pattern of the ciprofloxacin from the developed nano-in-micro hydrogel particles was determined by transferring a certain weight (10-30 mg) of particles to a vial containing 1.5 mL of PBS, pH 7.4. Samples were maintained at 37^{o}C with shaking at 100 rpm. At predetermined intervals, 100 μL aliquot was withdrawn and analyzed at λ_{max} 280 nm using a UV-Vis spectrophotometry. The withdrawn aliquots were replaced with the same volume of fresh buffer, to keep the volume of the release medium constant. The amount of ciprofloxacin released (μg) from the swellable particles was then calculated using a standard curve of ciprofloxacin in PBS, pH 7.4. Results were expressed as cumulative release (%) relative to the initially loaded weight of ciprofloxacin in particles. The data points represent average (with standard deviation) from three independent experiments.

4.3.2.8 Cytotoxicity assay of micro hydrogel particles

The effect of the developed swellable ciprofloxacin-loaded nano-in-micro hydrogel particles on the viability of RAW 264.7 cells was investigated. Cells were seeded in 96-well plates at 50,000 cells/well and incubated for 24 h at 37^{o}C and 5% CO_2. Fifty microliters of particles suspension (the total powder concentrations were 320, 800 and 1600 μg/mL, respectively.) were incubated with cells for 24 h. Control group was the cells grown without adding swellable particles and the cytotoxicity of plain PEG-g-NPHCs was also tested. The cell viability was estimated using a Microtiter tetrazolium

(MTT) cell proliferation assay kit provided by ATCC (Manassass, VA). After addition of the MTT reagent (10 µL), cells were incubated at 37°C and 5% CO_2 for 4 h until the purple precipitate was visible. Afterwards, 100 µl of detergent reagent was added and cells were left in the dark at room temperature for 2 h. Cell viability was determined through recording absorbance at 570 nm. Data represents average absorbance of triplicate samples (with standard deviation).

4.3.2.9 Preliminary in vivo pharmacokinetic studies

Experimental design: Male Sprague-Dawley rats weighing 350 ± 30g were obtained from the Charles River, and maintained in 12 h light/dark cycle. Rats were acclimated for 3 days before the experiment and were allowed free access to standard food and water. Temperature and relative humidity were maintained at 25 ℃ and 50%, respectively. All animal procedures were approved by the *Institutional Animal Care and Use Committee (IACUC)* of The University of Texas at Austin. All experiments related to animals were performed in according with the American Association for Accreditation of Laboratory Animal Care.

Male Sprague-Dawley rats were randomly assigned to 2 groups, receiving formulation as follows: group 1, the powder mixture of micronized ciprofloxacin with lactose (ciprofloxacin concentration: 30% w/w); group 2, dry swellable ciprofloxacin-loaded nano-in-micro hydrogel particle (ciprofloxacin concentration: 30% w/w). The dosage of ciprofloxacin was around 15mg/kg.

Briefly, prior to dosing, all rats were anesthetized by IP injection of ketamine (75mg/kg) / xylazine (5mg/kg) cocktail. The footpads were pinched firmly to test the lack of pedal reflex. Each animal was placed flat on its back and the trachea was visualized

with the help of a laryngoscope. The insufflator (Penn-Century DP-4, Penn Century, Philadelphia, PA) was inserted into the trachea. The dry powder in the chamber of insufflator was dispersed with the help of 2 mL of air from an empty syringe. After insufflation, each animal was held in an upright position for 1 minute to ensure appropriate deposition of powder in the lung, then maintained at 30° during the recovering period on the heating pad. The insufflator containing the powder was weighed before, after powder filling, and after administration, to know the exact dose insufflated.

4.3.2.10 Analysis of ciprofloxacin concentration in plasma, lavage and lung tissue

For sampling after administration, a 0.4 mL blood sample was collected from the jugular vein. As for the lavage collection, animals were euthanized via CO_2 inhalation and exsanguinated. The trachea was severed below the glottis between the thyroid glands, and was immediately cannulated and the lung was lavaged three times with 1 mL PBS (pH 7.4). The collected lavage was put in a 2 mL tube and kept in ice until analysis. Following lavage collection, the lung was removed, weighed and placed into a 50 mL conical tube, snap frozen and stored at -80 ºC until homogenization. For homogenization, lung tissues were thawed and 2 mL of PBS (pH 7.4) was added to each 50 mL tube. Lung was homogenized and the subsequent tissue slurry was analyzed for drug content.

The concentrations of the ciprofloxacin in the plasma, lavage and lung tissue were measured by using a high performance liquid chromatography (HPLC) system with an UV detector at room temperature. An aliquot of sample was injected into an HPLC column (Atlantis T3 Column, 4.6 x 250 mm, 5 µm, Waters Corporation, MA, USA). The mobile phase consisted of a mixture of 800 mL of 50 mL/L acetic acid, 110 mL of

acetonitrile, and 90 mL of methanol per liter. The sample was detected under 280 nm with **a** flow rate of 1mL/min at room temperature.

4.3.2.11 Statistical analysis

The obtained data was analyzed and expressed as mean with standard deviation. Effects of various parameters were statistically analyzed by one-way ANOVA. Differences were considered significant at the level of $p < 0.05$.

4.4 RESULTS AND DISCUSSION

4.4.1 Preparation of the swellable ciprofloxacin-loaded nano-in-micro hydrogel particles

One challenge for efficient pulmonary drug delivery is the lung physiological barriers (i.e., mucociliary escalator and alveolar macrophages), which should be considered thoroughly for the design of efficient pulmonary drug delivery systems. The preferred pulmonary delivery formulation should also possess controlled release profile along with minimal inactive excipients.

In order to overcome the challenges, a novel antibiotic hydrogel particle with high drug loading was formed for controlled pulmonary drug delivery. As well known, alginate can interact with Ca^{2+} to form hydrogel. However, Ca^{2+} can stimulate immune effects as mentioned previously. Instead of using Ca^{2+}, this study utilized ciprofloxacin, an antibiotic drug, as the cross-linker interacting with alginate directly and thereafter forming a high ciprofloxacin-loaded hydrogel particle system. In this high drug loading formulation, one fraction of ciprofloxacin was incorporated into PEG-g-PHCs nanoparticles; one fraction of ciprofloxacin was incorporated as a cross-linking agent for

alginate to form hydrogel, while the last fraction was physically entrapped in this hydrogel matrix (Scheme 1.). Hence, ciprofloxacin initially exhibited a burst release and reached a quick C_{max} due to free ciprofloxacin physically entrapped in the hydrogel matrix. Meanwhile, the other fractions of ciprofloxacin acting as the cross-linker, as well as the ciprofloxacin encapsulated in the nanoparticles, were sustained, which has the potential to control drug release in lungs.

The PEG-g-PHCs copolymer was synthesized through a modified method described in details in our previous study (9, 15). As illustrated in Scheme 2, the copolymer synthesis was achieved through firstly a phthaloylation process of the free amino groups of Cs to produce PHCs. FTIR spectrum of the PHCs (15) illustrated absorbance bands at 1395 and 732 cm^{-1} which were assigned for the aromatic C=C and C-H bonds of phthaloyl groups, respectively. Secondly, m-PEG was converted to m-PEG-COOH using succinic anhydride. The modification of m-PEG was confirmed using EA and FTIR (15). Conjugation of m-PEG-COOH with PHCs was then carried out and the obtained PEG-g-PHCs was also characterized using various analytical techniques (15).

The synthesized PEG-g-PHCs amphiphilic copolymer was utilized to prepare ciprofloxacin-free and ciprofloxacin-loaded self-assembled nanoparticles using sonication technique. Then, the resulting ciprofloxacin-loaded PEG-g-PHCs nanoparticles were incorporated into respirable micro hydrogel particles. These micro hydrogel particles were obtained via spray drying of a homogenous mixture of the ciprofloxacin-loaded PEG-g-PHCs nanoparticles and the aqueous alginate solution. The resulting nano-micro matrices were evaluated as carriers for sustained pulmonary

delivery of ciprofloxacin (ciprofloxacin concentration: 30% w/w) that combined the benefits of both nanoparticles and the micro hydrogel particles suggested in our previous studies (21).

Calcium, a divalent ion with two positive charges, interacts with alginate linear chains to form a cross-linked hydrogel (28). This mechanism of crosslinking has been studied and been reported elsewhere (23). Recently, we found ciprofloxacin, similar to calcium, can also act as a cross-linker with alginate to form a stable hydrogel. Although ciprofloxacin is zwitterionic, different from calcium which is divalent, the charges of ciprofloxacin could be well adjusted. Ciprofloxacin has three pKa values, pKa1=5.1, pKa2=6.4 and pKa3=9.0 (37). Therefore, at a pH lower than 5, the ciprofloxacin molecule has two positive charges, similar to ionic calcium. In our hydrogel formation experiment, ciprofloxacin was kept in a solution with pH less than 5.

4.4.2 Particle size

The size of the prepared ciprofloxacin-loaded nanoparticles was found to be 218.6 ± 25.3 nm as determined by DLS. In the case of the prepared micro hydrogel particles, the volume mean diameters (VMD) of plain microparticles and ciprofloxacin-loaded nano-in-micro hydrogel particles were determined using laser diffraction and were found to be 2.1 ± 0.1μm, and 3.9 ± 0.1 μm, respectively. The developed particles showed relatively low tapped densities (0.298 g/mL and 0.347 g/mL for plain microparticles and ciprofloxacin-loaded nano-in-micro hydrogel particles, respectively). The theoretical aerodynamic diameters (d_a) of both ciprofloxacin-free and ciprofloxacin-loaded particles were also calculated using the volume diameters and particle densities and were found to be 1.2 ± 0.7 μm and 2.3 ± 0.1 μm respectively. Although the low d_a values of these

prepared particles would lead to high particles respirability the powder dispersion studies showed only modest aerosol performance of the pure powders. The powder dispersion performance of this dry powder, without added carrier particles, was assessed *in vitro* by a commercial inhaler Handihaler® via the Next generation impactor (NGI, MSP Corp, MN) at air flow rate of 60 ± 5%L/min. The fraction of powder deposited in each stage of NGI, capsule, device, adaptor, and throat was determined. It was found that respirable fraction (RF%) and fine particle fraction (FPF%) were 21.5% and 28.6%, respectively. The dispersibility of the pure powders was similar to currently marketed product performance values and was encouraging given the absence of any other carrier particle (i.e. lactose) which is well known to enhance the dispersibility of micronized powders (38) .

In the control group, raw ciprofloxacin was jet-milled to achieve an X_{50} of 4.2 ± 0.5 μm. The X_{50} is the median diameter of the particles on a volume basis (as determined by laser diffraction). The jet milling procedure was optimized such that this particle size could match the particle size of the dried swellable hydrogel particles. The swellable particles with ciprofloxacin in dried form had an X_{50} of 4.6 ± 0.1 μm.

As a result of the similar particle size distribution of micronized ciprofloxacin and dried hydrogel particles, they represent well matched treatment and control groups in terms of particle size. In addition, insufflation, the method of directly spraying the dispersed powders into the lower airways of the animals, enables a direct comparison of the groups. This method of administration diminishes deposition differences between the two groups because the insufflator is placed intratracheally which avoids upper airway filtering on the basis of aerodynamic particle sizes. Thus, the deposition site of the two

kinds of particles in peripheral lung region after trachea insufflation should be similar. Therefore, after insufflation, differences in absorption and clearance of the two drug particles should be strongly related to particle size differences after wetting and swelling of the dried swellable particle, rather than the different deposition site of the two kinds of drug particles in the airways.

4.4.3 Surface morphology

Figure 1 shows the scanning electron micrographs of the developed micro hydrogel particles encapsulating ciprofloxacin-free and ciprofloxacin-loaded PEG-g-PHCs nanoparticles. As apparent from figure 1A, the ciprofloxacin-free hydrogel particles were generally spherical with relatively smooth surfaces. However, for the ciprofloxacin loaded particles, their surfaces were notably rougher (Fig 1B). The differences in surface roughness may be attributed to the additional crosslinking that occurs between the ciprofloxacin (for drug loaded particles) and the negatively charged sodium alginate chains during the spray drying process.

4.4.4 Dynamic swelling study

The swelling profile of the micro hydrogel particles incorporating ciprofloxacin-loaded PEG-g-PHCs self-assembled nanoparticles in PBS, pH 7.4 was shown in Figure 2. The swelling profile of the developed hydrogel particles was obtained through determining the increase in the volume mean diameter (VMD, μm) of the particles at various time intervals using laser diffraction technique. As shown in Figure 2, the prepared hydrogel particles showed a fast initial swelling within the first few minutes. For instance, the VMD of the developed particles has increased from about 3 μm when dry to 22 μm after 2 minutes of swelling. This swelling continued regularly with time to

reach 41.9 µm at 14 minutes. The swelling can be attributed to the hydrophilic nature of the PEG side chains in the PEG-g-PHCs copolymer and the sodium alginate. As the swelling behavior demonstrated, the micro developed particles which had respirable aerodynamic sizes when dry showed large geometric sizes when swollen after a short period in simulated moist environment of the lung. We have studied this behavior in similar particles at shorter time scales and have also confirmed that particle swelling occurs slower than the calculated transport time of the particle in the high humidity of the airways. This enables the particles to remain small enough during their passage to the deep lung where they will be deposited. However, the swelling that occurs on the order of minutes, as shown in Figure 2, enables the ciprofloxacin delivery systems to avoid macrophage uptake and at the same time confer sustained release of ciprofloxacin through a controlled polymeric architecture (9, 15, 20).

4.4.5 *In vitro* cumulative release study

The entrapment efficiency of ciprofloxacin in the developed swellable particles as quantified using UV-Vis spectrophotometry was found to be 30 % w/w. The *in vitro* release profile of ciprofloxacin from the developed swellable particles was illustrated in Figure 3. It was found that the investigated formulation showed a rapid initial release of ciprofloxacin (about 9%) within the first 5 hours followed by a relatively slow release up to 144 hours. The fast initial release may be due to the fast initial dynamic swelling of the investigated hydrogel particles as described in section 3.4. Several reasons **are** ascribed to the relatively slow and low release of the drug from the particles as observed in this *in vitro* assay. Firstly, a significant proportion of the ciprofloxacin present in the formulation will be bound to the alginate matrix and serves as a cross-linker. Therefore,

displacement of the ciprofloxacin by smaller monovalent cations will be necessary to enable release. In addition, the conditions of the release media were not selected to mimic physiological environment (i.e. release was performed in PBS pH 7.4). It is anticipated that more extensive release could be achieved in *in vivo* studies.

4.4.6 Cytotoxicity assay

The cytotoxicity of particles was determined using the MTT assay after the exposure of RAW 264.7 cells for 24 h to ciprofloxacin-loaded and plain PEG-g-PHCs at different concentrations (Figure 4). From the figure, at powder concentrations (i.e. 320 μg/mL) of ciprofloxacin-loaded nano-in-micro hydrogel particles in which the ciprofloxacin and PEG-g-PHCs concentration were both around 106.6 μg/mL , the viability of the RAW 264.7 cells was reduced by 38.4% as compared to the control cells under the same experimental conditions. This reduction in cell viability might be primarily attributed to the drug (ciprofloxacin) rather than the excipients in particles since the viability of cells cultured with drug-free PEG-g-PHCs at 1000 μg/mL was 93.6%.

4.4.7 Preliminary in vivo pharmacokinetic studies

Plots of average ciprofloxacin plasma concentration versus time after administration of two dry powder formulations to male Sprague-Dawley rats were shown in Figure 5. As apparent from the figure, there was a statistical difference in plasma concentrations of ciprofloxacin when comparing the swellable particle formulation with the powder mixture formulation at all-time points ($p < 0.05$). The pharmacokinetic parameters were calculated by non-compartmental methods (39). For plasma, the areas under the curve ($AUC_{0\text{-}7h}$) were 2.8 μg*h/mL, and 11.6 μg*h/mL for the group of swellable particles and the powder mixture group, respectively. For the T_{max}, even no

exact value of T_{max} could be achieved due to the limitation of time points, an estimation within the range of 0.25hr to 3 hr was determined. Overall, the mixture formulation showed much higher ciprofloxacin absorption into the plasma compared to the swellable particles, providing evidence for a delayed release from the swellable particles.

In Figure 6, the drug concentration measured in lung lavage fluids was not significantly different between the two formulation groups at the initial time point (t = 0.25 hr). As expected, with time going on lavage fluid drug concentrations decreased for both formulations. However, most notably ciprofloxacin decrease was observed in drug-lactose binary mixtures. This rapid decrease could be explained by different amount of dissolved drug available for absorption. Compared to swellable particles for controlled drug release, there would be more drugs dissolved from simply drug-lactose mixture group, ready for absorption into lung tissue and/or plasma. Additionally, micronized drug in powder mixture group was engulfed easily by macrophages. Subsequently, the engulfed ciprofloxacin would be transported along the respiratory surface to the mucociliary escalator or to lung interstitium and lymph nodes (40-42). The low bioavailability of powder mixture group was also confirmed by the AUC results. As calculated from Figure 6, the ratio of AUC_{0-7h} in lavage for swellable particles and control group was 1468.3 ± 377.5 μg*h/mL and 592.6 ± 139.9 μg*h/mL, respectively. These AUC data indicated that the swellable particles had significantly greater exposure to the lung fluid ($p = 0.04$) than the control group.

In *in vivo* lung biodistribution studies, the drug concentrations in lung lavage are related to the unreleased drug. Therefore, in our studies, ciprofloxacin in lavage sample was regarded as unreleased drug from the dry swellable hydrogel particles. Based on this

assumption, we determined that over 50% of drug was released from the swellable particles *in vivo*. Specifically, it was estimated that approximately 50% of the drug had been released after 0.25 hours, and approximately 80% at 3 hours.

Ciprofloxacin concentrations were also determined in the lung tissue samples. Lung concentrations were found to be higher for the swellable particles group than in the physical mixture group during the experimental period shown (Figure 7). The higher lung tissue concentrations from swellable particles could be attributed to two main factors. Firstly, the swellable particles formulation may extend the adhesion time between particles and lung epithelial cells, decreasing the particle clearance of mucociliary escalator in respiratory tract. Secondly, the release of the ciprofloxacin nanoparticles from the micro hydrogel particles can explain differences in the lung tissue levels. Nanoparticles may be more likely to be accumulated in the epithelial cells than free drug (as evidenced by the rapid plasma absorption of the free ciprofloxacin formulation). Further studies are required to determine the exact mechanisms of lung tissue distribution. Finally, the slightly different aerosol dispersion characteristics of the two treatment powders may also result in differences in deposition profiles (although this was minimized using the direct insufflation technique used in these studies) but may lead to small differences absorption and clearance from the lung.

4.8 CONCLUSION

In the present study, the self-assembled ciprofloxacin nanoparticles were encapsulated a dry swellable nano-in-micro hydrogel particles which were free of Ca^{2+}. This formulation displayed suitable aerodynamic characteristics and sustained drug release profile. When delivered to rats, it enabled ciprofloxacin to achieve a low systemic

exposure but maintained higher concentrations in the lung for more than seven hours. Further formulation optimization and studies focused at the cellular mechanisms of absorption and clearance are required.

4.9 ACKNOWLEDGEMENTS

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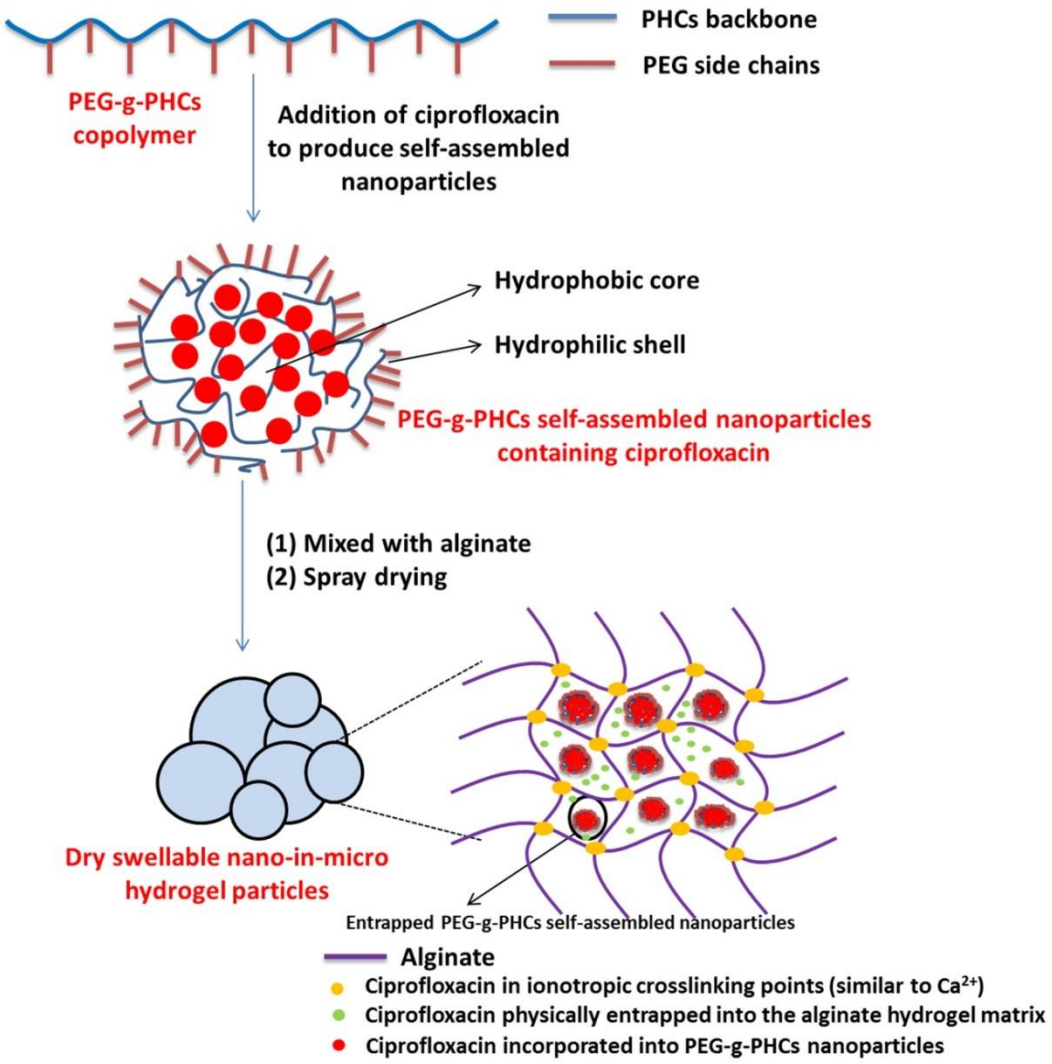
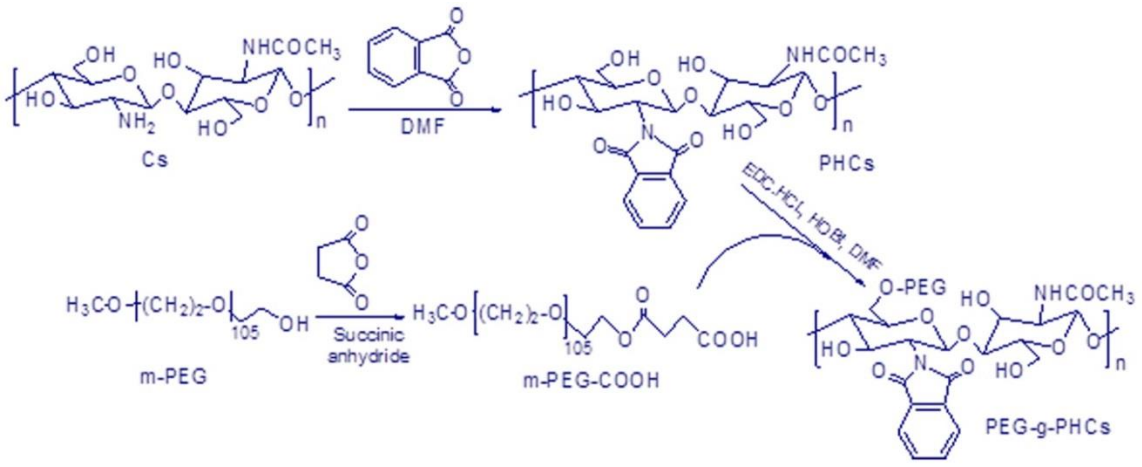
4.10 SCHEMES



Scheme 4.1 A schematic illustration for preparation of the dry swellable nano-in-micro hydrogel particles.



Scheme 4.2 Synthesis of PEG-g-PHCs amphiphilic copolymer

4.11 FIGURES

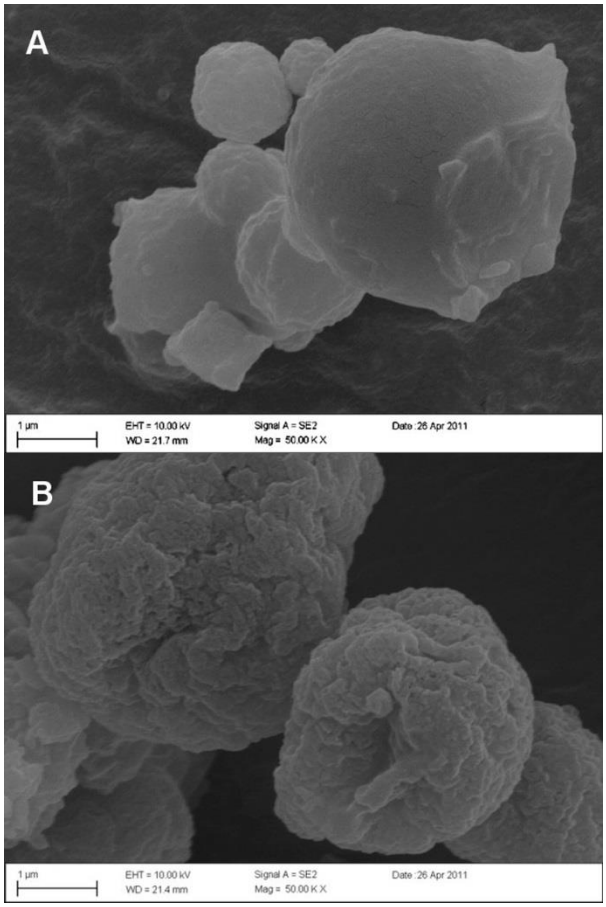


Figure 4.1 Scanning electron micrographs of (a) plain microparticles (no ciprofloxacin); (b) ciprofloxacin-loaded nano-in-micro hydrogel particles.

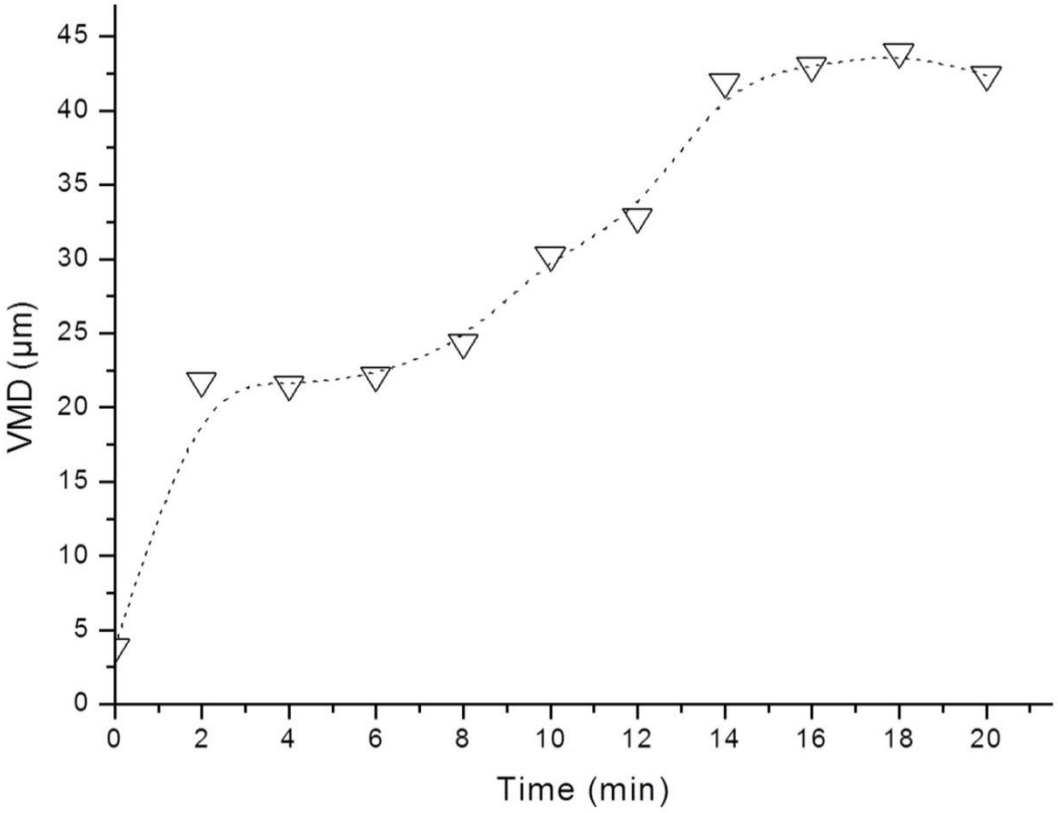


Figure 4.2 Dynamic swelling pattern of the swellable nano-in-micro hydrogel particles in PBS, pH 7.4.

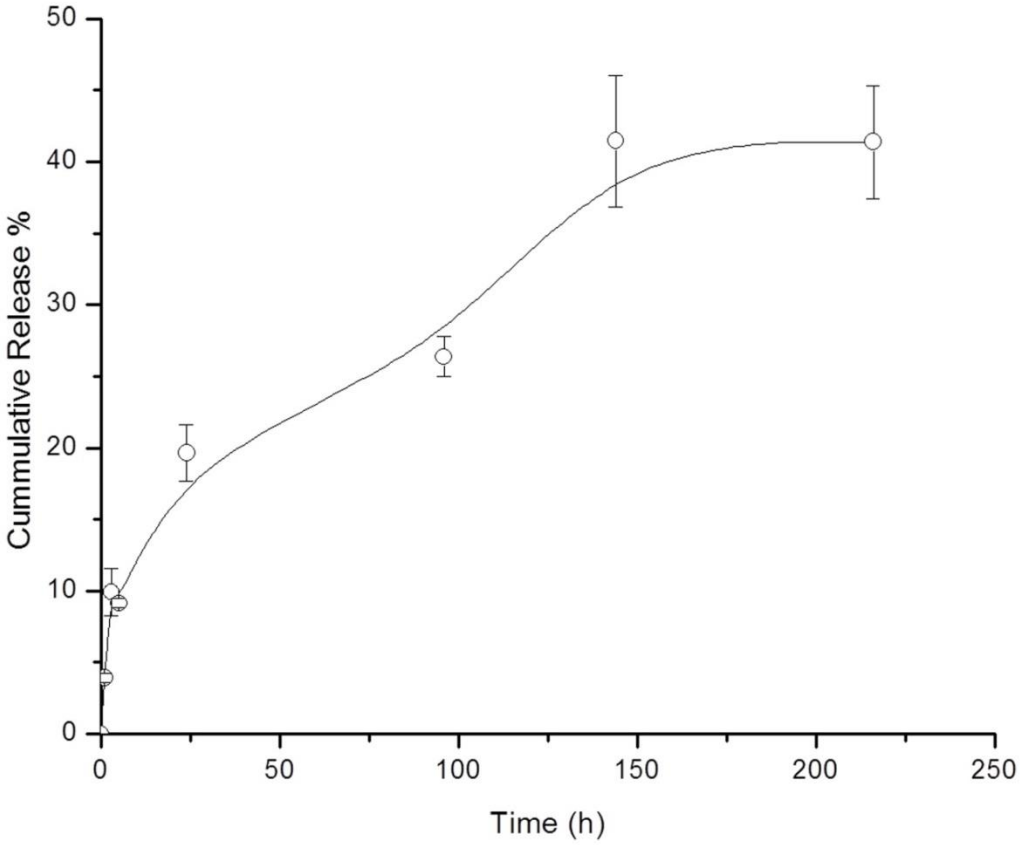


Figure 4.3 *In vitro* cumulative release of the ciprofloxacin from swellable nano-in-micro hydrogel particles.

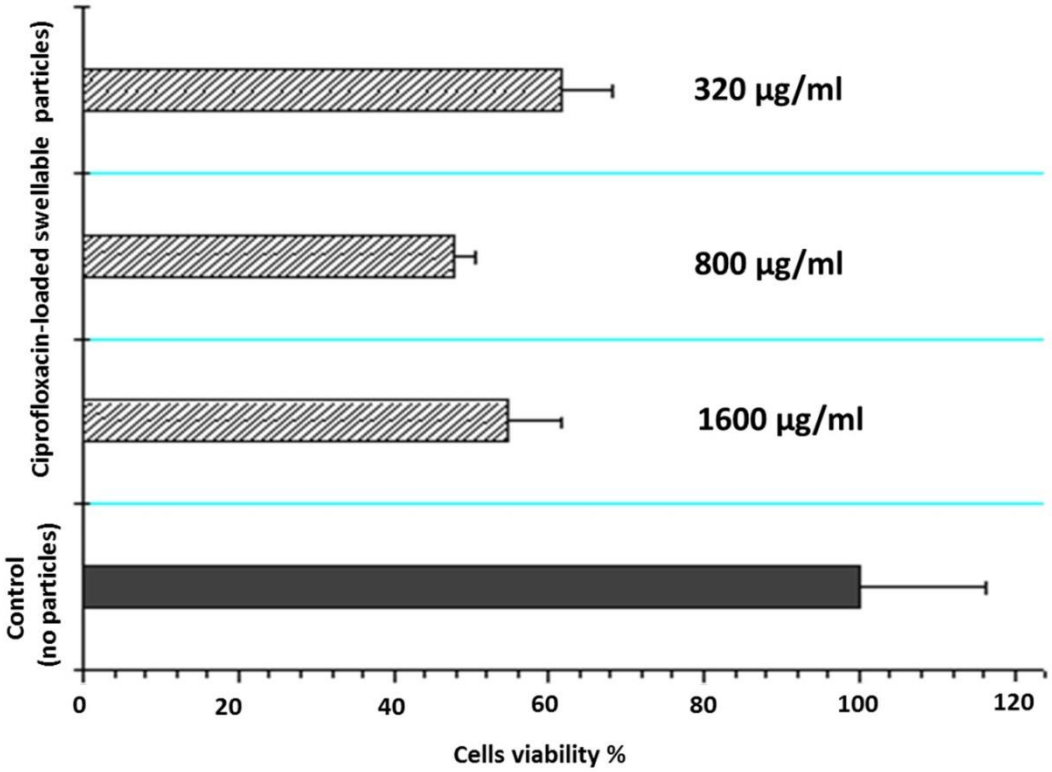


Figure 4.4 The effect of different concentrations (320, 800 and 1600 µg/mL) of the developed swellable ciprofloxacin-loaded nano-in-micro hydrogel particles on the viability of RAW 264.7 macrophage cells. Cells were seeded at 50,000 cells/well and incubated with the particles for 24 h at 37^{o}C and 5% $CO_{2.}$

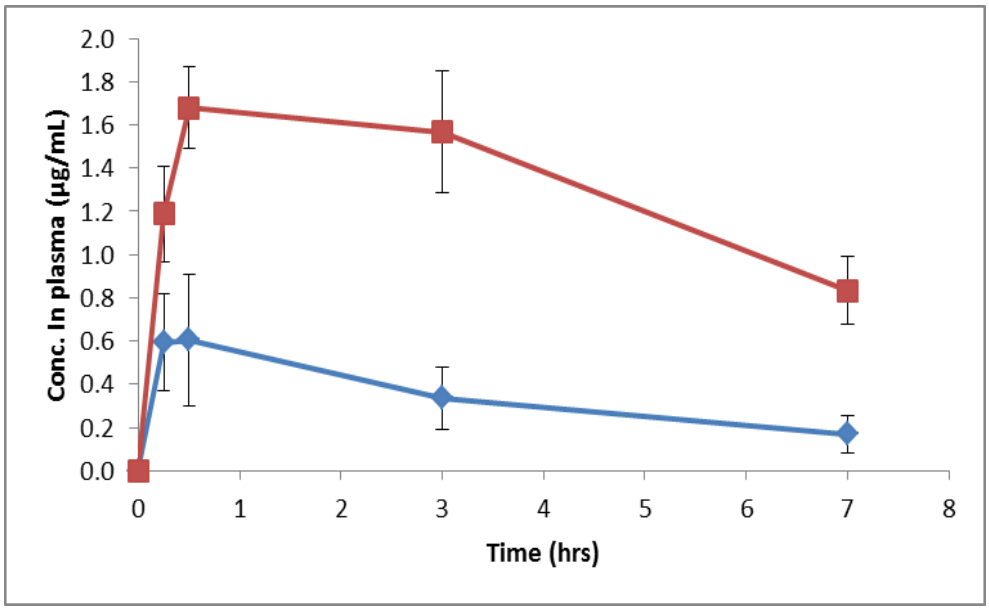


Figure 4.5 Time-course of concentration of ciprofloxacin in plasma. (♦),swellable ciprofloxacin-loaded nano-in-micro hydrogel particles; (■), powder mixture of micronized ciprofloxacin and Lactose (n=3-5). The dosage of ciprofloxacin was 15mg/kg.

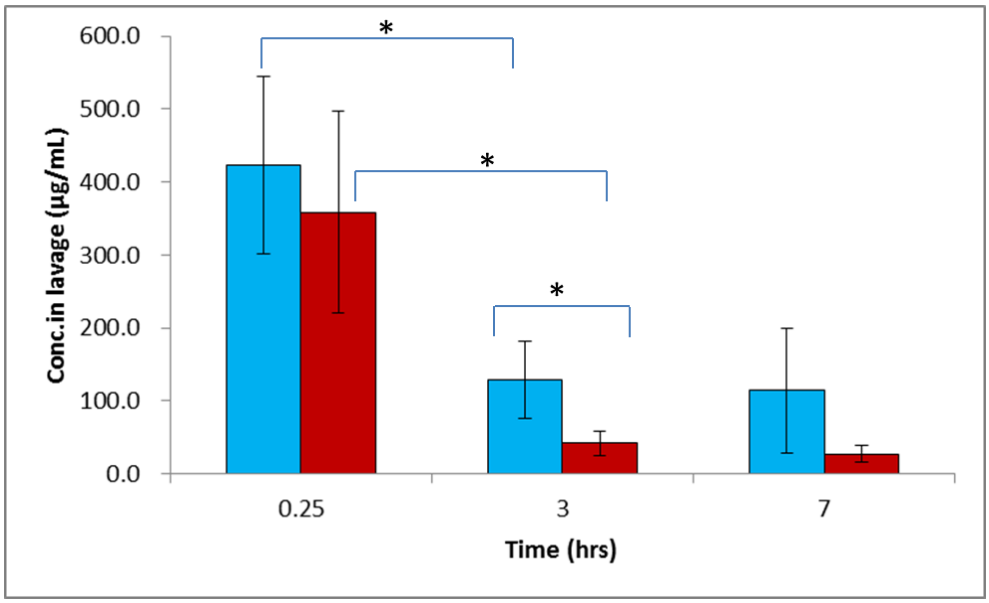


Figure 4.6 The concentration of ciprofloxacin in lung lavage. (■), swellable ciprofloxacin-loaded nano-in-micro hydrogel particles; (■), powder mixture of micronized ciprofloxacin and Lactose (n=3-5; *, $p<0.05$). The dosage of ciprofloxacin was 15mg/kg.

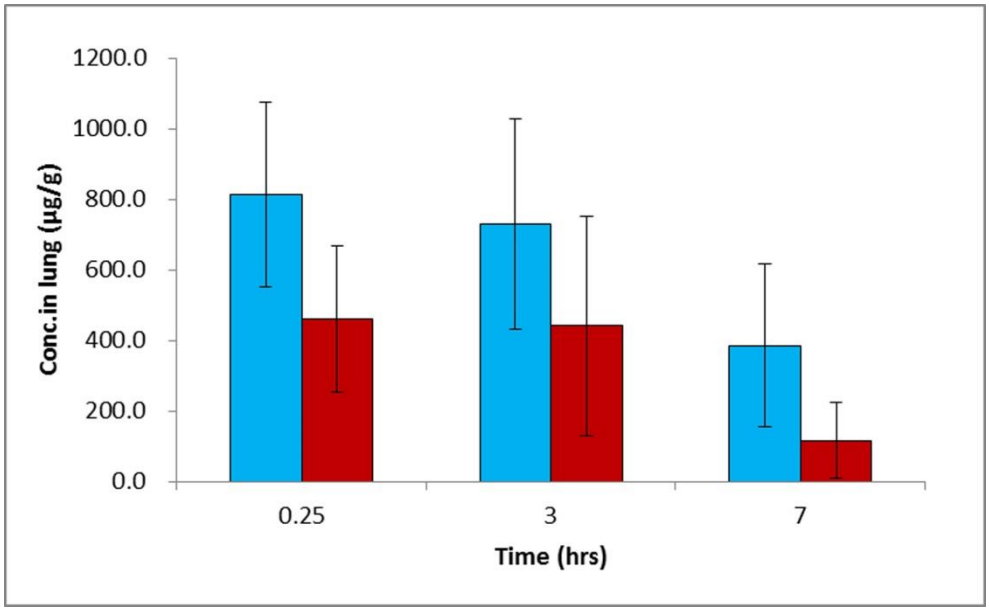


Figure 4.7 The concentration of ciprofloxacin in rat lung tissue. (■),swellable ciprofloxacin-loaded nano-in-micro hydrogel particles; (■), powder mixture of micronized ciprofloxacin and Lactose (n=3-5). The dosage of ciprofloxacin was 15mg/kg.

4.10 REFERENCES

1. Geller DE. Aerosol antibiotics in cystic fibrosis. Respir Care. 2009;54(5):658-70.

2. Pilcer G, Sebti T, Amighi K. Formulation and characterization of lipid-coated tobramycin particles for dry powder inhalation. Pharmaceut Res. 2006;23(5):931-40.

3. Weers JG, Bell J, Chan H-K, Cipolla D, Dunbar C, Hickey AJ, et al. Pulmonary formulations: what remains to be done? J Aerosol Med Pulm Drug Deliv. 2010;23(S2):S-5-S-23.

4. Yang Y, Tsifansky MD, Wu C-J, Yang HI, Schmidt G, Yeo Y. Inhalable antibiotic delivery using a dry powder co-delivering recombinant deoxyribonuclease and ciprofloxacin for treatment of cystic fibrosis. Pharmaceut Res. 2010;27(1):151-60.

5. Stass H, Weimann B, Nagelschmitz J, Rolinck-Werninghaus C, Staab D. Tolerability and Pharmacokinetic Properties of Ciprofloxacin Dry Powder for Inhalation in Patients With Cystic Fibrosis: A Phase I, Randomized, Dose-Escalation Study. Clin Ther. 2013;35(10):1571-81.

6. Ruge CA, Kirch J, Lehr C-M. Pulmonary drug delivery: from generating aerosols to overcoming biological barriers—therapeutic possibilities and technological challenges. Lancet Respir Med. 2013.

7. Bailey MM, Berkland CJ. Nanoparticle formulations in pulmonary drug delivery. Med Res Rev. 2009;29(1):196-212.

8. Houtmeyers E, Gosselink R, Gayan-Ramirez G, Decramer M. Regulation of mucociliary clearance in health and disease. Eur Respir J. 1999;13(5):1177-88.

9. El-Sherbiny IM, McGill S, Smyth HD. Swellable microparticles as carriers for sustained pulmonary drug delivery. J Pharm Sci. 2010;99(5):2343-56.

10. Wanakule P, Liu GW, Fleury AT, Roy K. Nano-inside-micro: disease-responsive microgels with encapsulated nanoparticles for intracellular drug delivery to the deep lung. J Control Release. 2012.

11. Du J, Du P, Smyth HD. Hydrogels for controlled pulmonary delivery. Ther Deliv. 2013;4(10):1293-305.

12. Dellamary LA, Tarara TE, Smith DJ, Woelk CH, Adractas A, Costello ML, et al. Hollow porous particles in metered dose inhalers. Pharmaceut Res. 2000;17(2):168-74.

13. Mansour HM, Rhee Y-S, Wu X. Nanomedicine in pulmonary delivery. Int J Nanomedicine. 2009;4:299.

14. Rytting E, Nguyen J, Wang X, Kissel T. Biodegradable polymeric nanocarriers for pulmonary drug delivery. 2008.

15. El-Sherbiny IM, Smyth HD. Biodegradable nano-micro carrier systems for sustained pulmonary drug delivery:(I) self-assembled nanoparticles encapsulated in respirable/swellable semi-IPN microspheres. Int J Pharm. 2010;395(1):132-41.

16. Byron PR. Prediction of drug residence times in regions of the human respiratory tract following aerosol inhalation. J Pharm Sci. 1986;75(5):433-8.

17. Patton JS, Byron PR. Inhaling medicines: delivering drugs to the body through the lungs. Nat Rev Drug Discov. 2007;6(1):67-74.

18. Tsapis N, Bennett D, Jackson B, Weitz DA, Edwards D. Trojan particles: large porous carriers of nanoparticles for drug delivery. Proc Natl Acad Sci U S A. 2002;99(19):12001-5.

19. Selvam P, El-Sherbiny IM, Smyth HD. Swellable hydrogel particles for controlled release pulmonary administration using propellant-driven metered dose inhalers. J Aerosol Med Pulm Drug Deliv. 2011;24(1):25-34.

20. El-Sherbiny IM, Smyth HD. Poly (ethylene glycol)–carboxymethyl chitosan-based pH-responsive hydrogels: photo-induced synthesis, characterization, swelling, and in vitro evaluation as potential drug carriers. Carbohyd Res. 2010;345(14):2004-12.

21. El-Sherbiny IM, Smyth HD. Controlled release pulmonary administration of curcumin using swellable biocompatible microparticles. Mol Pharm. 2011;9(2):269-80.

22. Kim D-H, Martin DC. Sustained release of dexamethasone from hydrophilic matrices using PLGA nanoparticles for neural drug delivery. Biomaterials. 2006;27(15):3031-7.

23. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog Polym Sci. 2012;37(1):106-26.

24. Gåserød O, Smidsrød O, Skjåk-Bræk G. Microcapsules of alginate-chitosan–I: a quantitative study of the interaction between alginate and chitosan. Biomaterials. 1998;19(20):1815-25.

25. Sechriest VF, Miao YJ, Niyibizi C, Westerhausen–Larson A, Matthew HW, Evans CH, et al. GAG-augmented polysaccharide hydrogel: A novel biocompatible and

biodegradable material to support chondrogenesis. J Biomed Mater Res. 2000;49(4):534-41.

26. Bhattarai N, Gunn J, Zhang M. Chitosan-based hydrogels for controlled, localized drug delivery. Adv Drug Deliver Rev. 2010;62(1):83-99.

27. Augst AD, Kong HJ, Mooney DJ. Alginate hydrogels as biomaterials. Macromol Biosci. 2006;6(8):623-33.

28. Sikorski P, Mo F, Skjåk-Bræk G, Stokke BT. Evidence for egg-box-compatible interactions in calcium-alginate gels from fiber X-ray diffraction. Biomacromolecules. 2007;8(7):2098-103.

29. Chan G, Mooney DJ. Ca< sup> 2+</sup> released from calcium alginate gels can promote inflammatory responses< i> in vitro</i> and< i> in vivo</i>. Acta Biomater. 2013;9(12):9281-91.

30. Orive G, Ponce S, Hernandez R, Gascon A, Igartua M, Pedraz J. Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginates. Biomaterials. 2002;23(18):3825-31.

31. Soon-Shiong P, Otterlie M, Skjak-Braek G, Smidsrod O, Heintz R, Lanza R, et al., editors. An immunologic basis for the fibrotic reaction to implanted microcapsules. Transplant P; 1991.

32. Clayton H, London N, Colloby P, Bell P, James R. The effect of capsule composition on the biocompatibility of alginate-poly-1-lysine capsules. J Microencapsul. 1991;8(2):221-33.

33. Otterlei M, Østgaard K, Skjåk-Bræk G, Smidsrød O, Soon-Shiong P, Espevik T. Induction of cytokine production from human monocytes stimulated with alginate. J Immunother. 1991;10(4):286-91.

34. Zimmermann U, Klöck G, Federlin K, Hannig K, Kowalski M, Bretzel RG, et al. Production of mitogen-contamination free alginates with variable ratios of mannuronic acid to guluronic acid by free flow electrophoresis. Electrophoresis. 1992;13(1):269-74.

35. Lee J, Lee KY. Local and sustained vascular endothelial growth factor delivery for angiogenesis using an injectable system. Pharmaceut Res. 2009;26(7):1739-44.

36. Edwards DA, Hanes J, Caponetti G, Hrkach J, Ben-Jebria A, Eskew ML, et al. Large porous particles for pulmonary drug delivery. Science. 1997;276(5320):1868-72.

37. Lin C-E, Deng Jr Y, Liao W-S, Sun S-W, Lin W-Y, Chen C-C. Electrophoretic behavior and p< i> K</i>< sub> a</sub> determination of quinolones with a piperazinyl substituent by capillary zone electrophoresis. J Chromatogr A. 2004;1051(1):283-90.

38. Smyth HD, Hickey AJ. Carriers in drug powder delivery. American Journal of Drug Delivery. 2005;3(2):117-32.

39. Sung JC, Padilla DJ, Garcia-Contreras L, VerBerkmoes JL, Durbin D, Peloquin CA, et al. Formulation and pharmacokinetics of self-assembled rifampicin nanoparticle systems for pulmonary delivery. Pharmaceut Res. 2009;26(8):1847-55.

Chapter 5: Drug Cross-Linked Hydrogel Particles for Controlled Pulmonary Drug Delivery

5.1 ABSTRACT

The current antibiotic therapies for treating pulmonary infections are usually dosed multiple times per day. Despite wide interests in developing controlled aerosol formulations, successful developments of controlled pulmonary drug delivery have been limited. The objective of this study was to form controlled drug release hydrogel dry powder suitable for pulmonary administration where the drug itself was utilized as the hydrogel cross-linker. The benefits of this novel approach were that the hydrogel dry powder would be calcium free, have high drug loading, and release the drug in a sustained manner. Alginate-ciprofloxacin hydrogel was first developed, then spray dried to form a dry powder. The hydrogel dry powder was characterized by scanning electron microscopy, laser diffraction, X-ray diffraction, and transmission electron microscopy. *In vitro* drug release studies were performed in a Transwell® model. The aerosol performance of the dry powder was investigated using the next generation impactor. The alginate hydrogel dry powder system exhibited high ciprofloxacin loading (57%) and a geometric size of less than 5 μm. Ciprofloxacin was present in the amorphous state in the dry powder and was released in a controlled release manner relative to ciprofloxacin alone, i.e. 80% of drug released at 8 hours. The hydrogel dry powder also achieved a high fine particle fraction (above 45%) as determined by the *in vitro* aerosol performance study. A novel inhalable alginate hydrogel dry powder system was successfully formulated and this discovery indicates broader applications for other antibiotics in the treatment of lung infections.

5.2 INTRODUCTION

The pulmonary delivery of antibiotics is a promising approach for the treatment of local lung infections (1, 2). For the current marketed antibiotic formulations, however, the requirement of multiple drug administrations per day in order to achieve a therapeutic effect limits their applicability (3).To reduce administration frequency, controlled pulmonary release formulation is a strategy that can maintain an effective and consistent local drug concentration and therefore prolong the time period between doses (4, 5). Previously, several types of controlled pulmonary release formulations for antibiotic delivery have been investigated, such as liposomes (6) and polymer based microparticles (i.e. PLGA) (7). However, due to the similarity of lipid materials in liposomes to the cell membrane, the interaction of the liposome with the lung cells may potentially increase the drug toxicity to the lung cells.by interacting with the lung cells (8). In addition, for the PLGA formulation, the process of formulation production always involves an organic solvent, which gives rise to the concern of residual solvent in the final product (9). Most importantly, these particles with an optimal aerodynamic diameter range targeted to the alveolar region (i.e. $0.5<d_a<5$ μm) will be rapidly cleared by the alveolar macrophages. This is because the geometric diameters of these particles are usually less than 6 μm, which is the preferable size range for uptake by the alveolar macrophages (10).

The approach we employed in the current study was to form swellable hydrogel dry powder by utilizing the unique benefits of hydrogel: higher drug payload, larger geometric diameter after swelling, and sustained drug delivery (11-13). Hydrogel is a well-studied formulation in biomedical applications, and now begins its new era in pulmonary delivery (14). In the peripheral respiratory system, many alveolar

macrophages exist in the alveolar region. Alveolar macrophages can phagocytos exogenous particles as a self-defense system, which largely reduces the effectiveness of inhaled drug particles. Alveolar macrophages prefer to select particles with a geometric size range of between 0.5-5 μm, a size that overlaps with most size range of respirable particles (10). For hydrogel, its ability to easily hydrate will allow it expand and effectively avoid uptake by macrophages. Therefore, the critical issue of respiratory clearance in developing functional inhalation formations will be compromised by fabricating hydrogel in dry state (15).

Alginate hydrogel is most frequently prepared using ionic cross-linking with a divalent agent (i.e. Ca^{2+}) (16). However, a recent study revealed the potential side effect that the calcium ions in calcium alginate gels may up-regulate the IL-1β secretion from the surrounding tissues of injection sites in mice (17). To provide solution to this potential problem, we propose a method to form a novel alginate hydrogel that has no calcium. We hypothesize that ciprofloxacin, an antibiotic, can directly interact with alginate, playing the role of a cross-linker (instead of calcium) in order to form the alginate hydrogel. In addition, this type of hydrogel particle will show a size range small enough for deep lung delivery. Upon contacting the lung surface, the particles will rehydrate and swell. This enables the particles to undergo significant increases in their geometric size within the lungs, thus eluding rapid clearance by alveolar macrophages.

The aim of this study was to achieve a formulation with a high drug loading, the merits of no Ca^{2+} related toxicity, and a simplified manufacturing process. The formulation described in this paper can also release the drug in a sustained manner, as expected with a hydrogel. In this novel formulation, one part of ciprofloxacin will

disperse uniformly through the hydrogel matrix, and another part of ciprofloxacin will ionically interact with alginate chains causing them to polymerize. Based on the two different ways of holding the drug in the hydrogel, the alginate-ciprofloxacin hydrogel dry powder is able to release the drug from the hydrogel matrix in a sustained manner, thereby reducing the administration frequency.

5.3 MATERIALS AND METHODS

5.3.1 Formation of Alginate Hydrogel

Alginate hydrogel was prepared by first forming an alginate-ciprofloxacin gel suspension followed by high speed homogenization. A volume of 30 mL of ciprofloxacin HCl solution (1.6% w/v, Letco, Decatur, AL) was added to 150 mL of alginate solution (0.16% w/v, Protanal CR8223, FMC Biopolymer, PA). Deionized water was used to prepare each of the solutions. Immediately upon addition, the two components formed an amorphous gel. This suspended gel was then homogenized using a rotor stator homogenizer (polytron PT2000, Kinematica, Switzerland). The homogenizer was operated at 11,000 RPM for 20 minutes using an ice bath to minimize temperature increase. The resulting homogenized gel suspension comprised of ciprofloxacin HCl and sodium alginate at a weight ratio of 1.5:0.75. The pH of this suspension sample was also determined (Accumet®, Fisher Scientific). The resultant gels and controls were evaluated using light microscopy (Olympus BX 53, Center Valley, PA).

5.3.2 Competition Study of Binding Capacity to Alginate between Ciprofloxacin and Calcium

To understand the mechanism of alginate gelling in the presence of ciprofloxacin, a competitive binding study between ciprofloxacin and calcium ions (i.e., Ca^{2+}) was performed. In this study, a solution composed of both calcium chloride and ciprofloxacin HCl was added into alginate solution (0.5% w/v), followed by high speed homogenization (11000 rpm for 20 seconds). The mole ratio of calcium to ciprofloxacin in the suspension was varied from 0:1 to 6:1. The resultant suspensions were then centrifuged at high speed (10000 rpm for 5mins, Hettich 320R, MA) to collect the supernatant. The free ciprofloxacin concentrations present in the supernatant were determined with UV-Vis (Infinite® M200, Tecan, CA).

5.3.3 Spray Drying to Form Alginate-Ciprofloxacin Hydrogel Dry Powder

Sodium alginate (0.5% w/v) and ciprofloxacin HCl (1% w/v) solutions were prepared in deionized water. The ciprofloxacin solution was then added into the alginate solution to yield a weight ratio of 1.0:0.75 (ciprofloxacin:alginate) to form a gel suspension via high speed homogenization as previously described. The pH value of suspension was determined to be approximately 5.7. The gel suspension was subsequently spray dried to form dry powder (BÜCHI B-290, BÜCHI Labortechnik AG, Switzerland). Spray drying was performed under the following parameters: 135°C inlet temperature, 68°C outlet temperature, 357 L/h air flow, 35 m^3 aspiration rate, and a feed pump flow of 5-10 mL/min. Figure 1 illustrated the process of spray drying to form the alginate-ciprofloxacin hydrogel dry powder.

5.3.4 Scan Electron Microscopy (SEM)

The scanning electron microscopy (Supra 40VP, Zeiss, Germany) was used to visually assess the size and morphology of the alginate hydrogel dry powder. The coating conditions for the tested samples were 15 nm of Pd/Pt via sputter coating.

5.3.5 X-Ray Diffraction (XRD)

X-ray powder diffraction (XRD) studies were performed to investigate the crystal / amorphous forms of ciprofloxacin in the alginate-ciprofloxacin hydrogel dry powder. Samples were run on a Philips 1710 X-ray diffractometer equipped with a copper target and a nickel filter (Philips Electronic Instruments, Inc., Mahwah, NJ). The voltage and current of the equipment were set as 40 KV and 40 mA, respectively. Samples were placed on a metal cell and flattened by using a glass slide prior to XRD analysis. The 2θ angle, step size, and dwell time were 5−50°, 0.05°, and 2 seconds, respectively.

5.3.6 Transmission Electron Microscope (TEM)

A drop of the alginate-ciprofloxacin hydrogel dry powder suspended in ethanol was placed on a carbon-coated copper grid and then dried under room temperature. The uncoated sample was directly investigated by a transmission electron microscope (JEOL, 2010F, Peabody, MA).

5.3.7 Swelling Study of Alginate-Ciprofloxacin Hydrogel Dry Powder

The swelling of the alginate-ciprofloxacin hydrogel dry powder was studied by determining the increase of the median diameter (X_{50}, μm) of the hydrogel dry powder in deionized water after a 2 minute suspension period. The measurement was taken using a

laser diffractometer (SYMPATEC, Sympatec Gmbt, System Partikl-Technik, Germany). The size of hydrogel dry powder in ethanol was tested as control.

5.3.8 *In Vitro* Drug Release Study

To evaluate the release profile of ciprofloxacin from the alginate-ciprofloxacin hydrogel dry powder, a Transwell assay was used. In short, a predetermined amount of powder was placed on a semi-permeable polycarbonate membrane (12 mm diameter insert, 0.4 µm pore size) on the donor compartment of a Transwell® insert (Corning, NY). To ensure that the release profile was not influenced by the intrinsic solubility limit of ciprofloxacin in this study (200 µg/mL, (18)), 1 mL of PBS (pH 7.4) was applied to disperse the dry powder (approximately 250 µg of dry powder, equivalent to 130 µg of ciprofloxacin) on the donor compartment. The Transwell® receptor contained 8 mL of PBS. The Transwell® device was then incubated at 37 °C without stirring (19). Aliquots (0.2 mL) of the PBS were taken from the receptor compartment at different time points within the period of 0 to 8 hours. Another 0.2 mL of fresh PBS was immediately put back into the receptor compartment to maintain a constant volume of release medium. As a control group, 1 mL of spray dried ciprofloxacin dry powder suspension in PBS (125 µg/mL) was directly placed onto the donor compartment. The collected samples were tested via UV-Vis at 280 nm. In a similar study, deionized water was used as the release medium instead of PBS, while the operational conditions were kept the same.

5.3.9 *In Vitro* Aerosolization Study

10 (±1) mg of powder, filled in the size 3 Vcaps HPMC capsule (Capsugel, CA), were dispersed through a commercial inhaler Aerolizer® (Novartis, Switzerland) into a next generation cascade impactor (NGI) (Copley Scientific, UK) operated at a volumetric

flow rate of 60 $Lmin^{-1}$ and actuated for 4 seconds. Drug content collected at each stage from the NGI apparatus was assessed via UV–Vis absorption spectroscopy at 280 nm. Fine particle fraction (FPF) was defined as the drug mass (<5 μm) deposited in the NGI divided by the emitted dose.

5.3.10 Statistical Analysis

The obtained data were analyzed and expressed as means with standard deviations. Effects of various parameters were statistically analyzed by t-tests. Differences were considered statistically significant at the level of $p < 0.05$.

5.4 RESULTS

5.4.1 Formation of Alginate Hydrogel

As shown in Figure 2, after adding ciprofloxacin HCl solution droplets into alginate solution, an opaque gel formed around the introduced ciprofloxacin droplets. It was observed that over time the interior of the introduced droplet became increasingly opaque. In addition, the gels formed by the addition of the ciprofloxacin droplets became more dense than the solution and sedimented at the bottom of the vial. After the addition of all the ciprofloxacin solution, the gels were heterogeneously distributed in the alginate solution. Homogenization was used to uniformly distribute the gel and reduce the size of the gels.

As shown in Figure 3A, alginate was observed to be a clear solution prior to the addition of ciprofloxacin HCl. In contrast, when a specific amount of ciprofloxacin was added into the alginate solution (Figure 3B, the weight ratio of drug to alginate was 1.5/0.75), chain-like structures were observed. Multiple chains bundled together could

also be observed (highlighted in the Figure 3B). As the amount of ciprofloxacin added increased, the formation of these bundles was increasingly observed. As a control, Figure 3C showed the alginate solution adjusted to a pH of 5.5 with HCl to match the pH of the alginate-ciprofloxacin mixture in Figure 3B. At this low pH there are no observable structures without the addition of ciprofloxacin.

5.4.2 Competition Study of Binding Capacity to Alginate between Ciprofloxacin and Calcium

From the competitive binding study between ciprofloxacin and calcium ions (Figure 4), it was observed that approximately 60% of ciprofloxacin was encapsulated into the alginate hydrogel matrix when no calcium was present (i.e., the mole ratio of ciprofloxacin to calcium was 1.0:0). When the mole ratio of ciprofloxacin to calcium was 1.0:0.5, there was no significant difference in ciprofloxacin encapsulation compared to calcium free gels. However, as the ratio of calcium to ciprofloxacin increased, the encapsulation percentage of ciprofloxacin in the alginate hydrogel matrix decreased significantly.

5.4.3 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was conducted to characterize the morphology of this alginate hydrogel dry powder containing ciprofloxacin as cross-linker. Figure 5C showed the morphology of the alginate-ciprofloxacin hydrogel dry powder produced from the alginate-ciprofloxacin gel suspension. The dry powders were observed to be collapsed spheres with an irregular shape. The individual particles had smooth surfaces with a size of less than 5 μm. Figures 5A and 5B illustrated the morphology of raw ciprofloxacin and alginate, respectively.

5.4.4 X-Ray Diffraction

In Figure 6, line A represented the crystal peaks of raw ciprofloxacin HCl. Line D showed the spectrum of alginate-ciprofloxacin dry powder. In contrast to line A, the representative crystal peaks of ciprofloxacin HCl were not present, but replaced with a broad peak at 24° to 28° (line D). As a control, the physical mixture of alginate with ciprofloxacin (line C) showed similar peaks as raw ciprofloxacin (line A), while raw alginate did not show any peaks (line B).

5.4.5 Transmission Electron Microscopy

TEM was conducted to investigate the existence of crystal ciprofloxacin in the alginate-ciprofloxacin hydrogel dry powder. As shown in Figure 7A and 7B, the alginate-ciprofloxacin hydrogel dry powder did not contain ciprofloxacin in crystal form, but rather in an amorphous form on the scale of 10 nm. The presence of the amorphous form of ciprofloxacin in the hydrogel dry powder was also confirmed by the absence of an electron diffraction pattern illustrated in Figure 7C. The presence of diffraction circles in the electron diffraction patterns of TEM can confirm the existence of crystals in the tested materials (20).

5.4.6 Swelling Study of Alginate Hydrogel Dry Powder

The swelling profile of the alginate-ciprofloxacin hydrogel dry powders was investigated using laser diffraction. The initial diameter of the hydrogel dry powder before swelling was approximately 4.1 µm. After 2 minutes in deionized water, the maximum diameter of the hydrated particles was determined to be approximately 7.6 µm.

5.4.7 *In Vitro* Drug Release Study

The *in vitro* drug release profiles of ciprofloxacin from the alginate-ciprofloxacin hydrogel dry powder were illustrated Figure 8. In Figure 8A, deionized water served as the release medium. The initial release rate of ciprofloxacin at the 0.25 h time point was the same for the ciprofloxacin dry powder and the alginate-ciprofloxacin dry powder. At longer time points however, there was a more rapid release of ciprofloxacin from the ciprofloxacin dry powder when compared to the alginate-ciprofloxacin hydrogel dry powder. The alginate-ciprofloxacin dry powder resulted in a slow release pattern of ciprofloxacin.

Figure 8B shows the release profiles of ciprofloxacin in PBS release medium. The release pattern of ciprofloxacin dry powder in the PBS was similar to that in deionized water. However, an increased release profile was detected from alginate-ciprofloxacin dry powder in PBS in contrast to that in deionized water. For the ciprofloxacin dry powder, 90% of the ciprofloxacin was released by 8 hours with 50% released in 1 hour. For the alginate-ciprofloxacin hydrogel dry powder, the 50% release time was approximately 4 hours.

5.4.8 *In Vitro* Aerosolization Study

The *in vitro* aerosol performance of alginate-ciprofloxacin hydrogel dry powder was assessed. The fractions of powder deposited in each stage of NGI, device (capsule, Aerolizer®, adaptor), induction port were determined (Figure 9). It was found that the fine particle fraction (FPF %), mass median aerodynamic diameter (MMAD) and were 46.8% and 2.7 μm, respectively. In addition, a high retained dose was observed in the device.

5.5 DISCUSSION

Ciprofloxacin displays both concentration-dependent and time-dependent antibacterial effects (21). However the AUC/MIC ratio has been reported to be the most efficient method to evaluate the antibiotic activity of fluroquinolones (22). Therefore, a larger AUC obtained from the development of a controlled release formulation will potentially benefit the management of lung infections, particularly when the drug is locally administered to achieve a high drug concentration at the site of infection.

5.5.1 Mechanisms of Ciprofloxacin Mediated Gelling of Alginate.

Ciprofloxacin has three pKa values associated with its piperazinyle substituent structure, which are: pKa_1=5.1, pKa_2=6.4 and pKa_3=9.0 (23). Therefore, depending on the pH of the surrounding environment, ciprofloxacin will have three distinct charged species that are designated as ciprofloxacin$\cdot H_2^{2+}$, ciprofloxacin$\cdot H^+$, and ciprofloxacin$^-$, along with a zwitterionic species of ciprofloxacin$^-\cdot H^+$. The pH value of the gel suspension obtained following homogenization of the drug-alginate mixture (the weight ratio of drug to alginate is 0.75/0.75) was about 5.8, indicating that around 70% of ciprofloxacin would exist as ciprofloxacin$\cdot H^+$ (24). The mono-cationic charge in ciprofloxacin$\cdot H^+$ will interact with anionic charge in alginate (25). When more ciprofloxacin HCl was added to the alginate solution, the pH value in the suspension (Figure 2B, the weight ratio of drug to alginate is 1.5/0.75) would be decreased to 5.5, and the divalent cationic form of Ciprofloxacin$\cdot H_2^{2+}$ will appear (23). At this pH, the extra positive charges present on Ciprofloxacin$\cdot H_2^{2+}$ will interact with the negative charges present on the adjacent alginate chains. Therefore, under these conditions,

ciprofloxacin facilitates the formation of junction zones and the drug acts as a cross-linker instead of the traditional Ca^{2+} (Figure 2B).

We have previously reported a similar swellable hydrogel dry powder for controlled pulmonary drug delivery. In that research, ciprofloxacin was firstly encapsulated into PEGylated chitosan nanoparticles which were then spray dried with alginate to form swellable and inhalable nano-in-microparticles for pulmonary drug delivery. The encapsulation efficiency for loading ciprofloxacin into the PEGylated chitosan nanoparticles was approximately 15% (The total ciprofloxacin loading efficiency in the nano-in-microparticles was around 30%.), and the free ciprofloxacin functioned as a cross-linker and interacted with the alginate. Due to the low loading efficiency of ciprofloxacin in these chitosan nanoparticles, as well as the complex and costly process of making PEGylated chitosan, here we directly combined ciprofloxacin with alginate to form a hydrogel dry powder without the addition of chitosan. We simplified the preparation method for these hydrogel microparticles, decreased the potential risk that polymeric chitosan may pose to lung tissue, and increased the ciprofloxacin loading efficiency from 30% to 50% in the final microsized hydrogel dry powder.

5.5.2 Competition Study between Ciprofloxacin and Calcium

As seen in Figure 4, when the mole ratio of ciprofloxacin to calcium was 1.0:0.5, the Ca^{2+} ions could interact with the alginate to form an alginate-calcium hydrogel via binding to the sites in the alginate chains where no ciprofloxacin had attached. This alginate-calcium hydrogel trapped some free ciprofloxacin inside the hydrogel matrix, leading to less free ciprofloxacin in the supernatant after centrifugation. However, with

the addition of more calcium into the suspension, Ca^{2+} ions began to compete with ciprofloxacin for the binding sites in the alginate chains where ciprofloxacin was bound. The significant decrease of ciprofloxacin encapsulation in the alginate matrix indicates that the binding capacity of calcium to alginate is stronger than that of ciprofloxacin, resulting in more ciprofloxacin molecules in the supernatant after high-speed centrifugation.

5.5.3 X-Ray Diffraction

As indicated in line D, representing the alginate-ciprofloxacin hydrogel dry powder, from Figure 6, the representative crystal peaks of ciprofloxacin HCl disappeared and were replaced with a broad peak pattern at 24° to 28°. This suggests that the ciprofloxacin molecule exists in an amorphous state in the alginate-ciprofloxacin hydrogel dry powder (26). The amorphous state of ciprofloxacin in the hydrogel dry powder can be easily explained. Prior to spray drying process, one portion of ciprofloxacin, approximately 60%, binds molecularly to alginate chains via ionic interactions between the positive charges of ciprofloxacin and the negative charges of the alginate chains. The other portion of the ciprofloxacin molecules is uniformly dispersed in the suspension of the alginate-ciprofloxacin system. During the spray drying process, the ionic interaction between ciprofloxacin and alginate is not influenced by the short period of heat. Therefore, the ciprofloxacin molecules binding with the alginate chains still molecularly interact with the alginate matrix after spray drying. The uniformly dispersed ciprofloxacin in the suspension is distributed evenly throughout the entire final hydrogel dry powder, either dispersed within the hydrogel matrix or located on the surface of hydrogel dry powder, as shown in Figure 1. XRD spectra showed the broad

peak of amorphous ciprofloxacin and therefore, both groups of ciprofloxacin (bound and unbound) were molecularly scattered throughout the alginate hydrogel dry powders.

5.5.4 *In Vitro* Drug Release Study

As shown in Figure 8A, in the deionized water release medium, less than 20% of the ciprofloxacin was released from the alginate-ciprofloxacin hydrogel dry powder before 8 hours. The slow release pattern may be explained by the fact that deionized water contains many fewer ions than PBS buffer. Therefore, no ions in the deionized water release medium can replace the ciprofloxacin ions and bind to the alginate chains (27). Consequently, the ciprofloxacin ions will firmly bind to alginate chains, leading to a slow release pattern of ciprofloxacin from the ciprofloxacin-alginate hydrogel dry powder.

The initial release pattern of less than 10% of the drug is attributable to the portion of ciprofloxacin attaching to the particles' surfaces or dispersing in the regions that are close to the surface after spray drying. This portion of drug is released with the hydrogel's swelling process upon interacting with the release medium. Even though the hydrogel dry powder could swell in the deionized water, it still kept a spherical shape through the end of the drug release test, indicating that ciprofloxacin is capable to cross-link alginate chains for a long period of time. In the PBS release medium, sodium and potassium ions in the release medium could interfere with the ionic interaction between the positively charged ciprofloxacin and negatively charged alginate chains, or even replace the ciprofloxacin ions as crosslinkers. Because of that, the ciprofloxacin was released relatively quickly from the hydrogel dry powder in PBS compared to that in deionized water.

Simulated lung fluid would better mimic the real lung physiological conditions. In the simulated lung fluid, more ions are present, such as $PO4^{3-}$, $SO4^{2-}$, Ca^{2+}, etc., which could potentially influence the release pattern of ciprofloxacin from this dry powder formulation. More interestingly, the presence of Ca^{2+} in the simulated lung fluid will trigger the ciprofloxacin release from the dry powder due to the competition between the calcium ions and the ciprofloxacin. Oppositely, the Ca^{2+} will also cross-link the alginate chains via ionic interactions to form a calcium based hydrogel, which could tighten the alginate powder matrix and subsequently hinder the diffusion of ciprofloxacin from the inside of the powder to the outmost layer. This remains to be seen and future studies will focus on these different possibilities.

5.5.5 Consideration of Therapeutic Dosage for Controlled Pulmonary Delivery

Due to the advantages of the respiratory system for local and systemic drug delivery, controlled release pulmonary formulations are becoming attractive. However, there is no controlled-release formulation for inhalation approved by FDA. This is mainly ascribable to the untested long-term safety profile of many of the excipients that could potentially be used (28-30). In general, it is polymer excipients that contribute to a prolonged drug release profile. Thus, a large mass fraction of polymer in the controlled release dosage form may be administered daily to patients via inhalation to the lung region. However, few polymers are in the approved excipients list for inhalation usage. Therefore, the application of unapproved polymers in controlled release pulmonary formulations will require extensive toxicity testing (14).

Alginate is one of most commonly used natural polymers to form hydrogels in biomedical applications. However, despite the extensive evaluations both *in vitro* and *in*

vivo, there is still a deep concern regarding immunogenicity of alginate (27, 31). One concern is associated with the ratio of alginate blocks (32). Alginate is composed of unbranched copolymer that contains two blocks, the M-block and the G-block. It has been reported that alginate with a high ratio of M-block to G-block is more immunogenic, and therefore may trigger an increased release of cytokines than that with high ratio of G-block to M-block (33). In contrast, no immunogenicity was detected from alginate with varied levels of M-block (34). Due to the different sources or batches of alginate used, its immunogenicity may also be related to the impurities left during the extraction process from natural resources. Thus impurities such as heavy metals, endotoxins, proteins and polyphenolic compounds may be present in alginate, and these may cause an immune response (27). Therefore, highly purified alginate with fewer impurities can decrease its potential immunogenicity in biomedical applications. For example, no foreign body reaction was observed in animals when alginate was processed via a multi-step purification technique (31, 35). In the current study, alginate was crossed-linked with ciprofloxacin to form hydrogel designed for pulmonary delivery. More studies are ongoing in our lab to investigate the interaction between alginate polymer and respiratory cells.

5.6 CONCLUSION

In the present study, we developed an alginate-ciprofloxacin hydrogel dry powder that was free of Ca^{2+} and exhibited a high ciprofloxacin loading with a high fine particle fraction suitable for deep lung delivery. Upon interacting with moisture, this hydrogel dry powder in the dry state will rehydrate and swell to a larger geometric size therefore avoiding macrophages in the lungs. In addition, ciprofloxacin, as a cross-linker to interact

with alginate chains, was present in the amorphous state and exhibited a sustained released from the hydrogel dry powder.

5.7 ACKNOWLEDGEMENTS

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5.8 FIGURES

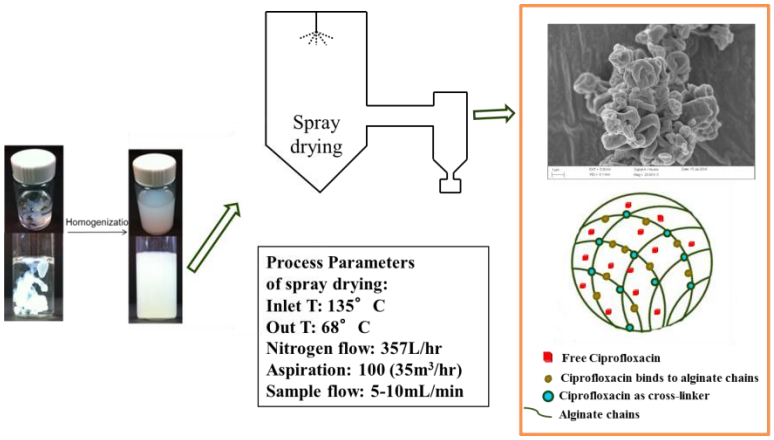

Figure 5.1 Diagram of the spray drying process used to form the alginate-ciprofloxacin hydrogel dry powder.

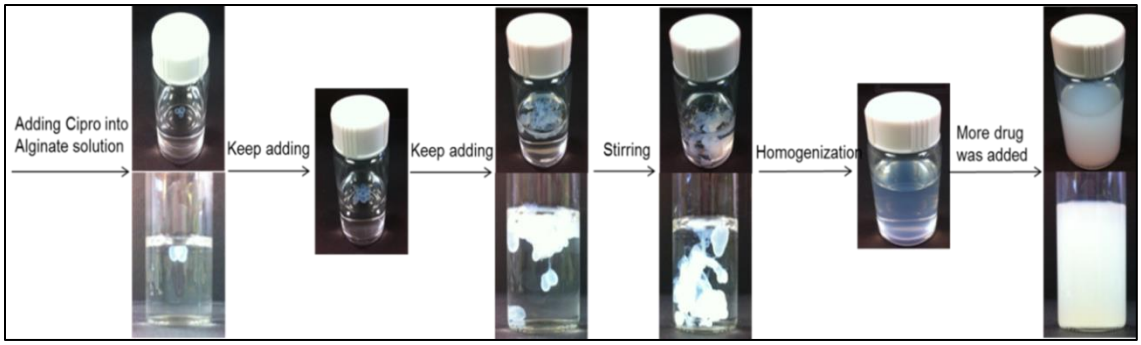

Figure 5.2 Gel formation between alginate and ciprofloxacin.

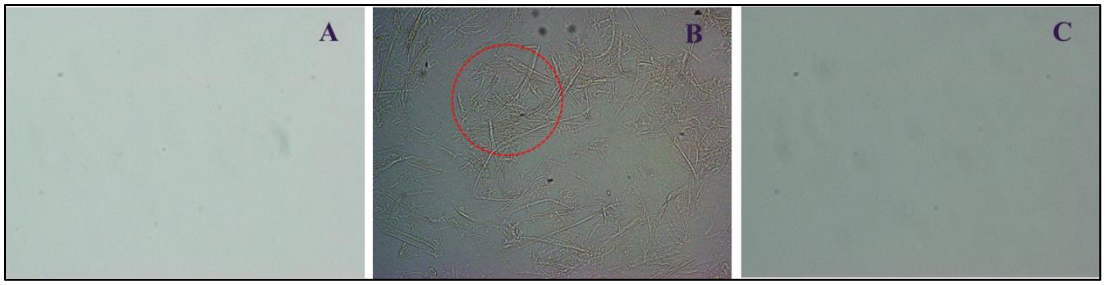

Figure 5.3 Microscopy of chain-like structures in the alginate-ciprofloxacin hydrogel system. A. Alginate solution when no ciprofloxacin was added; B. Suspension of alginate-ciprofloxacin system, the weight ratio of alginate to ciprofloxacin was 1.5:0.75, pH 5.5; C. Alginate solution with HCl adjusted to pH 5.5.

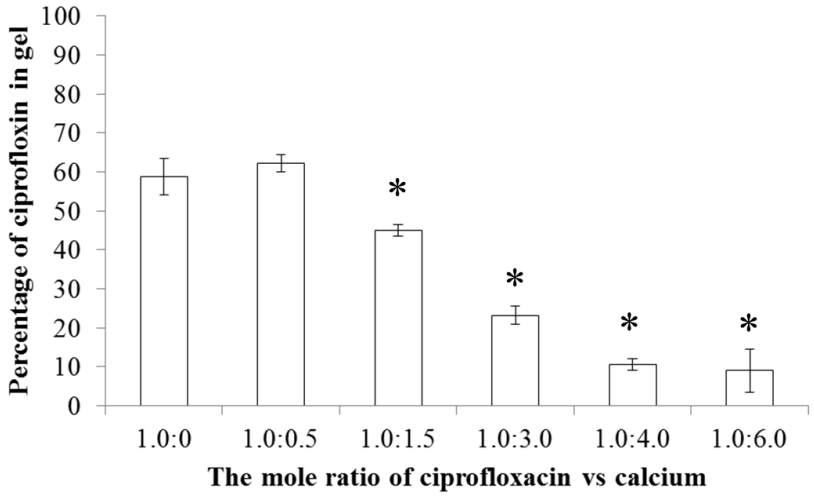

Figure 5.4 Competition study of binding capacity to alginate between calcium and ciprofloxacin; *, $p < 0.05$, t-test compared to the percentage of ciprofloxacin in the hydrogel suspension when the mole ratio of ciprofloxacin to calcium to was 1.0:0.

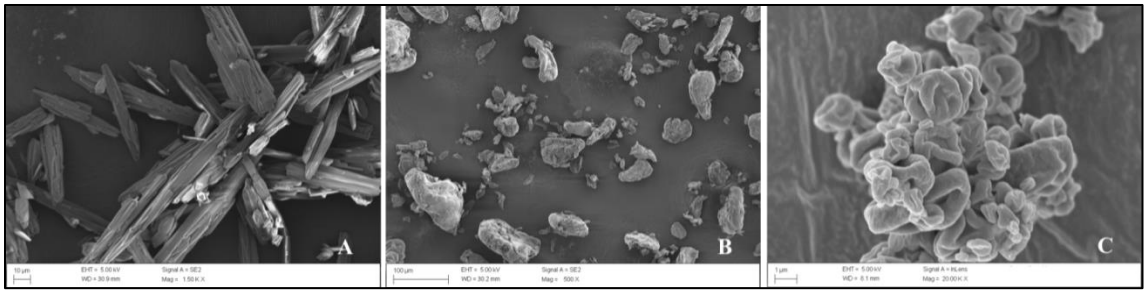

Figure 5.5 Scanning electron microscopy of spray dried alginate-ciprofloxacin hydrogel dry powder. A. Ciprofloxacin HCl crytals; B. Sodium alginate powder; C. Spray dried alginate-ciprofloxacin hydrogel dry powder (57% w/w of ciprofloxacin in dry powder).

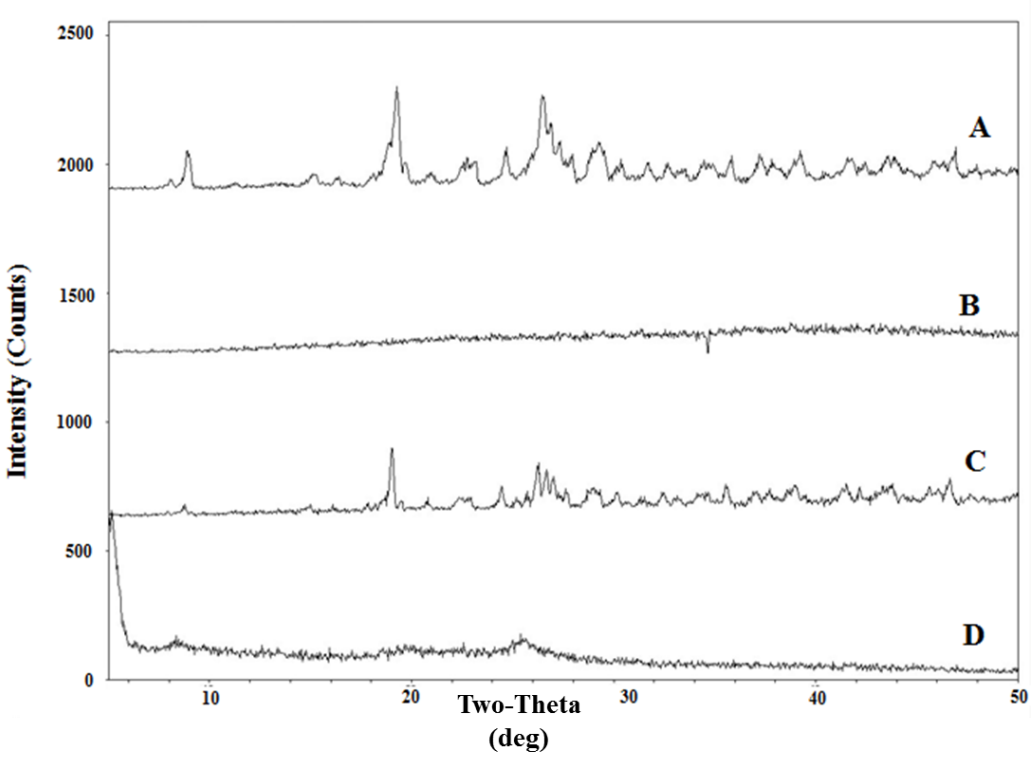

Figure 5.6 X-ray diffraction patterns of spray dried alginate-ciprofloxacin hydrogel dry powder. A. Ciprofloxacin HCl crystals; B. Sodium alginate powder; C. Mixture of ciprofloxacin HCl with sodium alginate powder (57% w/w of ciprofloxacin), D. Spray dried alginate-ciprofloxacin hydrogel dry powder (57% w/w of ciprofloxacin in dry powder).

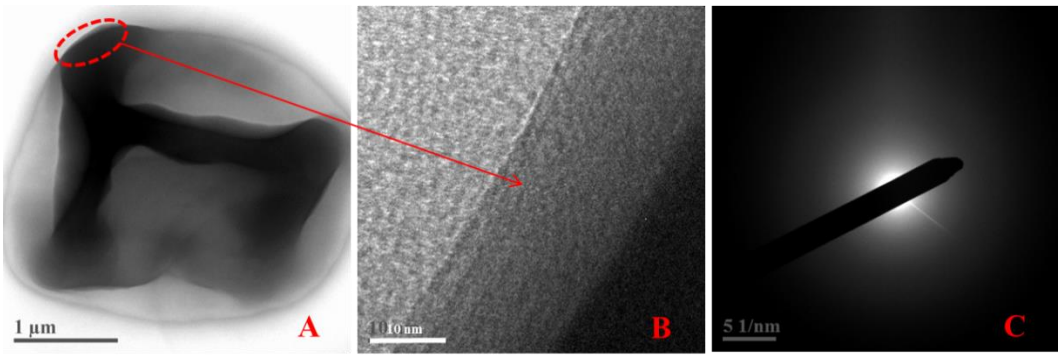

Figure 5.7 Transmission electron microscopy of spray dried alginate-ciprofloxacin hydrogel dry powder (57% w/w of ciprofloxacin in dry powder). A, B, C, were the alginate-ciprofloxacin hydrogel dry powder.

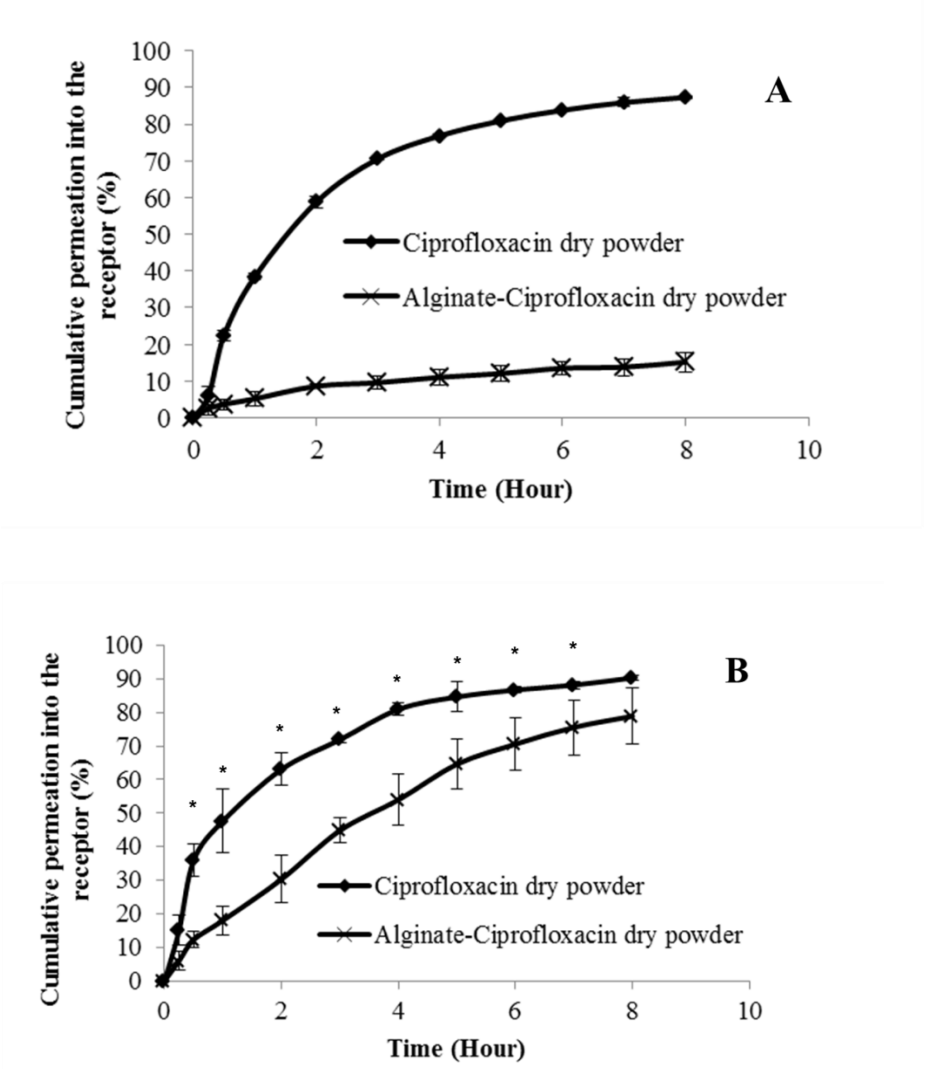

Figure 5.8 *In vitro* drug release profiles of Alginate-ciprofloxacin hydrogel dry powder. A. Drug release profile in deionized water B. drug release profile in PBS. *, $p < 0.05$ using a t-test

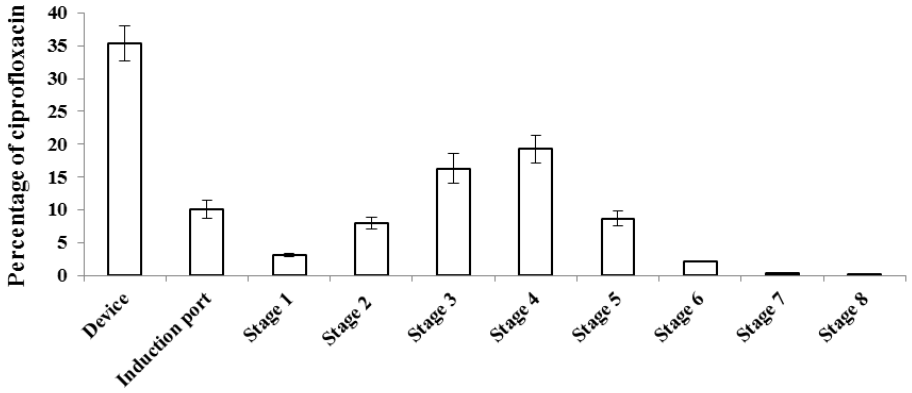

Figure 5.9 In vitro aerosol profile of spray dried alginate-ciprofloxacin hydrogel dry powder (57% w/w of ciprofloxacin in dry powder).

5.9 REFERENCES

1. Serisier D. Inhaled antibiotics for lower respiratory tract infections: focus on ciprofloxacin. Drugs of today (Barcelona, Spain: 1998). 2012;48(5):339-51.

2. Ibrahim BM, Tsifansky MD, Yang Y, Yeo Y. Challenges and advances in the development of inhalable drug formulations for cystic fibrosis lung disease. Expert opinion on drug delivery. 2011;8(4):451-66.

3. Weers JG, Bell J, Chan H-K, Cipolla D, Dunbar C, Hickey AJ, et al. Pulmonary formulations: what remains to be done? Journal of aerosol medicine and pulmonary drug delivery. 2010;23(S2):S-5-S-23.

4. Yildiz A, John E, Özsoy Y, Araman A, Birchall JC, Broadley KJ, et al. Inhaled extended-release microparticles of heparin elicit improved pulmonary pharmacodynamics against antigen-mediated airway hyper-reactivity and inflammation. Journal of Controlled Release. 2012;162(2):456-63.

5. Scalia S, Salama R, Young P, Traini D. Preparation and in vitro evaluation of salbutamol-loaded lipid microparticles for sustained release pulmonary therapy. Journal of microencapsulation. 2012;29(3):225-33.

6. Liu C, Shi J, Dai Q, Yin X, Zhang X, Zheng A. In-vitro and in-vivo evaluation of ciprofloxacin liposome for pulmonary administration. Drug development and industrial pharmacy. 2013(0):1-7.

7. O'Hara P, Hickey AJ. Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: manufacture and characterization. Pharmaceutical research. 2000;17(8):955-61.

8. Lasic D. Applications of liposomes. Handbook of biological physics. 1995;1:491-519.

9. Sahana D, Mittal G, Bhardwaj V, Kumar M. PLGA nanoparticles for oral delivery of hydrophobic drugs: influence of organic solvent on nanoparticle formation and release behavior in vitro and in vivo using estradiol as a model drug. Journal of pharmaceutical sciences. 2008;97(4):1530-42.

10. Wanakule P, Liu GW, Fleury AT, Roy K. Nano-inside-micro: Disease-responsive microgels with encapsulated nanoparticles for intracellular drug delivery to the deep lung. Journal of Controlled Release. 2012;162(2):429-37.

11. Kwon MJ, Bae JH, Kim JJ, Na K, Lee ES. Long acting porous microparticle for pulmonary protein delivery. International journal of pharmaceutics. 2007;333(1):5-9.

12. Surendrakumar K, Martyn G, Hodgers E, Jansen M, Blair J. Sustained release of insulin from sodium hyaluronate based dry powder formulations after pulmonary delivery to beagle dogs. Journal of Controlled Release. 2003;91(3):385-94.

13. Selvam P, El-Sherbiny IM, Smyth HD. Swellable hydrogel particles for controlled release pulmonary administration using propellant-driven metered dose inhalers. Journal of aerosol medicine and pulmonary drug delivery. 2011;24(1):25-34.

14. Du J, Du P, Smyth HD. Hydrogels for controlled pulmonary delivery. Therapeutic delivery. 2013;4(10):1293-305.

15. El-Sherbiny IM, Smyth HD. Controlled release pulmonary administration of curcumin using swellable biocompatible microparticles. Molecular pharmaceutics. 2011;9(2):269-80.

16. Haug A, Smidsrod O. The effect of divalent metals on the properties of alginate solutions. Acta Chem Scand. 1965;19(2).

17. Chan G, Mooney DJ. Ca< sup> 2+</sup> released from calcium alginate gels can promote inflammatory responses< i> in vitro</i> and< i> in vivo</i>. Acta biomaterialia. 2013;9(12):9281-91.

18. Yang Y, Tsifansky MD, Wu C-J, Yang HI, Schmidt G, Yeo Y. Inhalable antibiotic delivery using a dry powder co-delivering recombinant deoxyribonuclease and ciprofloxacin for treatment of cystic fibrosis. Pharmaceutical research. 2010;27(1):151-60.

19. Arora D, Shah KA, Halquist MS, Sakagami M. In vitro aqueous fluid-capacity-limited dissolution testing of respirable aerosol drug particles generated from inhaler products. Pharmaceutical research. 2010;27(5):786-95.

20. Allcorn E, Manthiram A. NiSb–Al2O3–C Nanocomposite Anodes with Long Cycle Life for Li-Ion Batteries. The Journal of Physical Chemistry C. 2014;118(2):811-22.

21. Roberts JA, Lipman J. Antibacterial dosing in intensive care. Clin Pharmacokinet. 2006;45(8):755-73.

22. Wright DH, Brown GH, Peterson ML, Rotschafer JC. Application of fluoroquinolone pharmacodynamics. J Antimicrob Chemoth. 2000;46(5):669-83.

23. Lin C-E, Deng Jr Y, Liao W-S, Sun S-W, Lin W-Y, Chen C-C. Electrophoretic behavior and p< i> K</i>< sub> a</sub> determination of quinolones with a piperazinyl substituent by capillary zone electrophoresis. Journal of Chromatography A. 2004;1051(1):283-90.

24. Aristilde L, Sposito G. Complexes of the antimicrobial ciprofloxacin with soil, peat, and aquatic humic substances. Environmental Toxicology and Chemistry. 2013;32(7):1467-78.

25. Stewart MB, Gray SR, Vasiljevic T, Orbell JD. The role of poly-M and poly-GM sequences in the metal-mediated assembly of alginate gels. Carbohydrate Polymers. 2014.

26. Mahmoudi ZN, Upadhye SB, Ferrizzi D, Rajabi-Siahboomi AR. In Vitro Characterization of a Novel Polymeric System for Preparation of Amorphous Solid Drug Dispersions. The AAPS journal. 2014:1-13.

27. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Progress in polymer science. 2012;37(1):106-26.

28. L C-C. In vivo animal models for controlled-release pulmonary drug delivery. Smyth HD, Hickey AJ, editors. NY US: Springer; 2011. 443-87 p.

29. Rossi SE, Erasmus JJ, McAdams HP, Sporn TA, Goodman PC. Pulmonary Drug Toxicity: Radiologic and Pathologic Manifestations 1. Radiographics. 2000;20(5):1245-59.

30. Singh GLP, G. P. Development and approval of inhaled respiratory drugs: a US regulatory science perspective. Smyth HD, Hickey AJ, editors. NY, US: Springer; 2011.

31. Orive G, Ponce S, Hernandez R, Gascon A, Igartua M, Pedraz J. Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginates. Biomaterials. 2002;23(18):3825-31.

32. Clayton H, London N, Colloby P, Bell P, James R. The effect of capsule composition on the biocompatibility of alginate-poly-1-lysine capsules. Journal of microencapsulation. 1991;8(2):221-33.

33. Otterlei M, Østgaard K, Skjåk-Bræk G, Smidsrød O, Soon-Shiong P, Espevik T. Induction of cytokine production from human monocytes stimulated with alginate. Journal of Immunotherapy. 1991;10(4):286-91.

34. Zimmermann U, Klöck G, Federlin K, Hannig K, Kowalski M, Bretzel RG, et al. Production of mitogen-contamination free alginates with variable ratios of mannuronic acid to guluronic acid by free flow electrophoresis. Electrophoresis. 1992;13(1):269-74.

35. Lee J, Lee KY. Local and sustained vascular endothelial growth factor delivery for angiogenesis using an injectable system. Pharmaceutical research. 2009;26(7):1739-44.

Chapter 6: Polyethylene Glycol Conjugated Tobramycin Improved Antimicrobial Activity in *P. aeruginosa* Biofilms[1, 2]

6.1 ABSTRACT

The eradication of microbial infections is extremely challenging due to the recalcitrant nature of the causative microbial biofilms. The objective of this study was to develop a functionally enhanced antibiotic that would improve the therapeutic activity against bacterial biofilms. Here we explore the hypothesis that by reducing the affinity of an antibacterial toward biofilms would result in improved efficacy of this antibacterial. To test this hypothesis, a conventional antibiotic, tobramycin, was chemically conjugated with 5000 kDa polyethylene glycol (PEG) via site specific conjugation to form a novel form of PEGylated-tobramycin (Tob-PEG). The antibacterial efficacy of Tob-PEG, as compared to that of the non-conjugated tobramycin, was assessed on the planktonic phase and biofilms phase of *Pseudomonas aeruginosa* (*P. aeruginosa*) using a standard biofilm assay. The minimum inhibitory concentration (MIC_{80}) of Tob-PEG was higher (13.9 μmol/L) than that of tobramycin (MIC_{80}, 1.4 μmol/L) in the planktonic phases of *P. aeruginosa*. In contrast, the Tob-PEG was approximately 3.2 fold more effective in eliminating bacterial biofilms than tobramycin. Specifically, Tob-PEG had minimum inhibitory concentrations lower than those exhibited by tobramycin (MIC_{80} 27.8 μmol/L vs 89.8 μmol/L). Confocal laser scanning microscope and scanning electron microscope findings further confirmed these data. Thus, modification of antimicrobial as by PEGylation (Tob-PEG) appears to be a promising approach for overcoming the bacterial resistance in the established biofilms of *P. aeruginosa.* The mechanisms by which the

conjugated Tob-PEG enhances the elimination of *P. aeruginosa* biofilm are currently being investigated.

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2. Statement of co-author contribution. This chapter was written by Ju Du; some sections were written by HMHN Bandara. Dr. Hugh Smyth helped with editorial and content assistance. The research idea was made by Ju Du. The synthesis of Tob-PEG was done by Ju Du. The *in vitro* bioactivity studies were performed by HMHN Bandara. Ping Du, Hui Huang, Khang Hoang, Dang Nguyen, Sri Vasudha Mogarala offered the research assistance to this work in the laboratory.

6.2 INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that most prominently affects the airways. This chronic and debilitating disease is caused by a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene and leads to an impaired mucociliary clearance mechanism(1). Due to this impaired clearance and subsequent susceptibility to infection, an inflammatory response, characterized by recruitment of polymorphonuclear leukocytes (PMN) and stimulation of antibody production (1, 2), is mounted. This increased inflammation then leads to further disease manifestation commonly described as the "downward spiral" in cystic fibrosis (3). Despite aggressive antibiotic treatment, the elimination of chronic *P. aeruginosa* infections in CF lungs is extremely difficult (4, 5). The pathogen often adapts to resist both the host inflammatory defense mechanisms and externally applied antibiotic therapy, often allowing for the formation of microbial biofilms (6-8).

Biofilms are complex, functional communities of one or more species of microorganisms that are encased in extracellular polymeric substances (EPS) and attached to both a solid surface and to each other (9). The effective dose of an antimicrobial needed to eliminate biofilms can be up to 1000 times greater than that of the planktonic phase (10, 11). The slow growth rates (12), low antibiotic penetration (13), high cell density (10), excessive extracellular matrices (14), pH alterations (15), mutations (16) and altered nutrient requirements (17) are well-known properties that cause this high antimicrobial resistance in microbial biofilms. *P. aeruginosa* usually starts colonizing in the airway in CF patients as a non-mucoid strain in their early stages of life. As the infection progresses, the pathogen switches to a virulent mucoid form that

secretes excessive amounts of EPS (18, 19), which consists of an abundance of polysaccharides, proteins, and DNA (20). The resulting biofilms are thick, pathogen embedded, and highly resistant to common therapeutic agents currently used in CF infections such as beta-lactams, ciprofloxacin, tobramycin and colistin (14, 21-29).

Tobramycin is a broad spectrum aminoglycoside antibiotic commonly used in treating *P. aeruginosa* infections (30, 31). Tobramycin binds the 30S and 50S subunits of bacterial ribosomes thereby preventing the formation of 70S ribosomes. As a result, bacterial mRNA cannot be translated into proteins, leading to microbial cell death (32). There are two FDA approved tobramycin formulations for the management of CF patients, including tobramycin inhalation solution (TOBI®) and tobramycin inhalation powder (TOBI® Podhaler™) (33, 34). Despite the broad-spectrum antibacterial effects and benefits of inhalation, resistance to tobramycin treatment in these patients occurs regularly (13, 19, 24-26, 35).

Thus, the development of newer antimicrobial agents with superior abilities to eliminate the established chronic biofilm associated with CF infections remains the utmost priority in CF therapy (36, 37). Our hypothesis, derived from the proposed mechanisms of biofilm resistances, is that reducing the binding or affinity of the antibacterial toward the biofilm itself will result in improved antibiotic efficacy. To test this hypothesis, a conventional antibiotic, tobramycin was chemically modified. Tobramycin has previously been demonstrated to bind to biofilm matrices (38), thus reducing the effective concentration of antimicrobial able to reach the pathogenic organisms, as well as limiting the penetration of the antibacterial agent to the deeper microstructure of the biofilm, thereby creating an undesirable stress response in the

pathogen (17, 39). It is essential to improve the penetrative capabilities of existing antimicrobials, such as tobramycin, in order to overcome thick biofilm barriers and to achieve superior elimination of *P. aeruginosa* biofilms. Modifying existing drugs by conjugating them to polymers has been widely reported to improve the efficacy of existing drugs (40-42). Predominantly, conjugation to polyethylene glycol (PEG) has been used to increase plasma half-lives of therapeutic agents (43, 44). PEGylation has also been shown to improve diffusion of nanoparticles through mucus (45). To our knowledge, the conjugation of PEG to tobramycin has not been reported in the literature, but its feasibility is supported by reports that have shown that chemical modification at the 6' amine group of tobramycin will still maintain antibacterial activity (46).

Thus, the aim of this study was to conjugate tobramycin with PEG and compare the antibacterial performance of this conjugate to tobramycin.

6.3 MATERIALS AND METHODS

6.3.1 Synthesis of Tob-PEG

Tob-PEG was prepared according to a modified method as shown in Figure 1 (47-49). Briefly, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1mmol, EDC·HCl, AnaSepc Inc., Fremont, CA, USA) and N-Hydroxysuccinimide (1 mmol, NHS, Aldrich, St. Louis, MO, USA) were added into a 50 mL triangular flask containing 15 mL PBS buffer with PEG-COOH (0.5 mmol, synthesized in our lab as described previously) (50) and stirred overnight at room temperature. After that, tobramycin sulfate (0.8 mmol, Letco, Decatur, AL, USA) was added and stirred overnight under similar conditions. The resulting product was dialyzed against distilled water and subsequently lyophilized. Nuclear Magnetic Resonance (NMR) spectra of the final product of Tob-PEG were recorded (Varian DirectDrive 400 spectrometers) at 400MHz in the NMR facility of the Department of Chemistry & Biochemistry of The University of Texas at Austin.

6.3.2 Microbial Culture

Pseudomonas aeruginosa (*P. aeruginosa*) PAO1 (kindly provided by Dr. Marvin Whiteley at The University of Texas at Austin) was used throughout the study. Blood agar (Sigma Aldrich, St. Luis, MO, USA) and Brain Heart Infusion (BHI, Sigma Aldrich, St. Luis, MO, USA) broth were used for culturing *P. aeruginosa*.

Prior to each experiment, *P. aeruginosa* were cultured on blood agar for 18 h at 37°C. BHI was inoculated and incubated for 18 h at 37°C in an orbital shaker (150 rpm, VWR® Incubating Orbital Shaker, VWR international, Radnor, PA, USA). The resultant

bacterial growth was harvested, washed twice with phosphate buffered saline (PBS, pH 7.4, Sigma Aldrich, St. Luis, MO, USA), and resuspended in BHI. The concentration of *P. aeruginosa* was adjusted to 1×10^7 cells/mL by spectrophotometer (Infinite M200, TECAN systems Inc., San Jose, CA, USA) and confirmed by hemocytometric counting.

6.3.3 Biofilm Formation

P. aeruginosa biofilm was formed as previously described with minor modifications (51). A commercially available sterile, polystyrene, flat bottom 96-well microtiter plate (BD Biosciences, CA, USA) was used throughout the biofilm studies. First, 100 µL of standard bacterial suspension (10^7 cells/mL) was transferred into each well of 96-well microtiter plate, and incubated for 90 min in an orbital shaker (37°C, 80 rpm) to promote microbial adherence to the wells. After the initial adhesion phase, each well was aspirated then was gently washed twice with PBS to remove loosely adherent bacterial cells. Then, 200 µL of BHI was added to each well, and the plate was incubated for 24 h in an orbital shaker (37°C, 80 rpm). Prior to evaluation of each therapeutic agent, supernatant in the each well was aspirated and the biofilm was washed twice with PBS to eliminate excess media.

6.3.4 Determination of Minimum Inhibitory Concentration (MIC_{80})

6.3.4.1 The P. aeruginosa Planktonic Phase

Bacterial suspension (5×10^5 cells/mL) in designated wells of a 96-well microtiter plate was treated with each therapeutic agent in a concentration gradient (2-fold dilution) and incubated for 24 h at 37°C. At the end of this incubation, bacterial growth was measured by optical density using a spectrophotometer at 595 nm. For each agent, the

lowest concentration at which 80% of bacterial growth was inhibited was considered the MIC_{80} against *P. aeruginosa.* Each experiment was conducted in quadruplicates on three different occasions.

6.3.4.2 The P. aeruginosa Biofilm Phase

P. aeruginosa biofilms were developed as described in the biofilm formation section. After 24 hours, biofilms were gently washed as mentioned above, and each test agent, including tobramycin, PEG, a physical mixture of PEG and tobramycin, Tob-PEG and PBS (as control) were added to the biofilms at various predetermined concentrations. The plates were then incubated with these test compounds for another 24h in an orbital shaker (37°C, 80 rpm). At the end of the incubation, an XTT reduction assay was performed to quantify the viability of the biofilms. This experiment was conducted in quadruplicates on three different occasions.

6.3.5 XTT Reduction Assay

After incubation of biofilms with each tested reagent, a standard XTT reduction assay was performed to measure the viability of biofilms by means of bacterial cell metabolic activity (52, 53). In brief, commercially available XTT powder (Sigma, St. Louis, MO, USA) was dissolved in PBS by 1 mg/mL, sterile filtered (0.22 μm pore size filter), and stored at -70°C. Fresh 0.4 mM menadione solution was prepared prior to experiment. Before the assay, XTT solution was thawed and mixed with menadione solution at 20:1 (v/v). Then, 158 μL of PBS, and 42 μL mixture of XTT and menadione solution were immediately added into each well containing bacterial biofilms, and allowed to incubate in the dark for 3 h at 37°C. The color intensity of the resultant solution was measured by a spectrophotometer at 492 nm.

6.3.6 Visual Alginate and Drug Interaction Study

A simple assay was developed to visually demonstrate the differences in interaction between the different drugs and formulations used in these performance studies. A volume of 50 µL alginate solution (0.5 g/100 mL) was applied to a glass slide. One drop of drug or control (tobramycin, PEG, unbound mixture of tobramycin with PEG, Tob-PEG) was added to the top of alginate solution on the glass slide using a micropipette. Immediately after applying the drop of drug solution, the slide was examined by light microscopy imaging at 15 times magnification on a Planapo 2.0X microscope (Leica M205 FA, Germany). All test samples were applied at a concentration of 1.4 mmol/L. An untreated alginate solution, PEG, and the physical blend of PEG and tobramycin were used as the control groups.

6.3.7 Confocal Laser Scanning Microscopy

P. aeruginosa biofilms were grown on glass cover slips placed in the bottom of 6-well plate (BD Biosciences, USA) as previously described (51). The biofilms were gently washed with PBS and treated with the test agents (tobramycin, PEG, Tob-PEG and PBS) at their respective MIC_{80} calculated for the biofilms,(51) and incubated for 24 h in an orbital shaker (37°C, 80 rpm). Biofilms on cover slips in each well were then stained with Live and Dead stain (Live/Dead BacLight Bacterial Viability kit, Invitrogen, Eugene, OR, USA) and imaged by confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems, IL, USA).

6.3.8 Scanning Electron Microscopy (SEM)

Biofilms were prepared for SEM as previously described (54). *P. aeruginosa* biofilms were formed on the glass cover slips in a 6-well plate and treated with the different test agents (PBS, tobramycin, PEG, and Tob-PEG) as previously described in the methods of confocal laser scanning microscopy. After a 24 h incubation, the cover slips were removed from the well, gently washed twice with PBS, and fixed with an aldehyde mixture in an ice bath for 3 h then were exposed to reduced osmium tetroxide in a microwave (2 min on, 2 min off, 2 cycles, Pleco Biowave, 100 w). The cover slips were then washed in distilled water, and dehydrated in a series of ethanol washes (50%, 70%, 95%, and 100%, 10 min each) and finally dried in a critical point dryer (> 40 $^{\circ}$C, > 1200 psi, Samdri-790 Critical point Dryer, Tousimis Research Co., MD, USA). Finally, the cover slips were coated with Platinum/Palladium (Cressington sputter coater 208 HR, Cressington Scientific Instruments Ltd, UK) and surface topography of the biofilms formed on the cover slip was visualized with scanning electron microscope (Zeiss Supra 40VP, CA, USA) in high-vacuum mode.

6.3.9 Statistical Analyses

During the determination of MIC_{80}, the broth dilution assay gave rise to the same specific drug concentration that caused 80% inhibition of *P. aeruginosa* at each of the biological and technical replicates, therefore generating a zero standard deviation. This is a common occurrence and has been previously reported in these types of assays (55-57).

6.4 RESULTS

6.4.1 Synthesis and Characterization of Tob-PEG Conjugate

Figure 2B shows representative H-NMR spectra for tobramycin, PEG, and Tob-PEG, illustrating the successful synthesis of the conjugated Tob-PEG. In Figure 2B, the multiplets at around 5.45 ppm were assigned to the H at A1', while multiplets at 4.90 ppm corresponded to the H at C1''. The two H at A3' had two correlations with the signals at about 2.10 ppm and 1.75 ppm, and the two H at B2 were linked with the peaks at about 2.05 ppm and 1.45 ppm. Compared to the H-NMR spectrum of tobramycin, the peaks from 1.4 ppm to 2.1 ppm shifted slightly to left in the H-NMR spectrum of Tob-PEG, which was due to the interference of the PEG_{5k}. The peaks at 5.50 ppm and 4.95 ppm in the H-NMR spectrum of Tob-PEG matched well with that of Tobramycin as described above. These characterized peaks of tobramycin were absent in the H-NMR spectrum of PEG_{5k}. Multiplets overlapped with each other within the range of around 3 ppm to 4 ppm, which was attributed to the polymeric structure of PEG.

6.4.2 Antibacterial Activity in the Planktonic and Biofilms

One of the major objectives of conjugation of tobramycin with PEG was to achieve superior antibacterial effects as compared to just tobramycin. However, according to the broth microdilution assay, tobramycin was still more effective than Tob-PEG in inhibiting the planktonic *P. aeruginosa* growth as manifested by the respective MIC_{80}, 1.4 μmol/L.

As revealed by an XTT reduction assay, which directly assessed bacterial cell metabolic activity, Tob-PEG demonstrated a significantly lower MIC_{80} against mature *P.*

aeruginosa biofilms when compared to tobramycin (27.8 µmol/L vs 89.8 µmol/L). Tob-PEG was at least 3 fold more efficient than tobramycin. Physically mixed tobramycin and PEG (ie. not conjugated) exhibited MICs similar to tobramycin alone (89.8 µmol/L) against mature biofilms. Chemical conjugation of tobramycin and PEG led to favorable antibacterial activities over tobramycin in mature biofilms.

6.4.3 Visual Alginate and Drug Interaction Study

As shown in Figure 4A, the microscope image of the control, untreated alginate solution was clear and transparent. In Figure 4B, the added drop of drug immediately produced a visible boundary between the added drug droplet and the alginate solution. This spherical formation did not dissipate with time. In Figure 4D, the mixture of non-conjugate PEG with tobramycin resulted in a similar visible interaction between the added drop and the alginate solution, though the boundary dispersed more than with tobramycin alone. In Figure 4C, however, the added droplet containing Tob-PEG did not result in a visible boundary. The same was observed for the added PEG solution (Fig. 4E).

6.4.4 Confocal Laser Scanning Microscopy

The mature *P. aeruginosa* biofilms, characterized using confocal microscopy, were densely colonized with hierarchically and three-dimensionally structured formations as shown in Figure 5A. Biofilms were observed to have a significant amount of extracellular polymeric substances with a high live/dead cell ratio (Fig. 5A). Even though the biofilms treated with PEG alone (Fig. 5B) visually appeared to be thinner, there were no significant changes observed when compared to the control biofilms (Fig. 5A). In contrast, mature biofilms treated with tobramycin at its MIC_{80} (for biofilm phase) showed a greater reduction of total biofilm with a scanty architecture. A higher proportion of

dead cells with lower quantities of extracellular materials were seen after treatment with tobramycin (Fig. 5C). Most significantly, the *P. aeruginosa* biofilm treated with Tob-PEG exhibited only a few isolated bacterial colonies instead of a recognizable biofilm structure. Additionally, no extracellular material was visible and the biofilm architecture was completely disrupted when compared to all other treated biofilms (Fig. 5D). Thus, these qualitative findings further confirmed that the newly synthesized Tob-PEG molecule possessed superior antibiofilm properties over traditional tobramycin.

6.4.5 Scan Electron Microscopy (SEM)

Despite the modification of biofilms caused by the necessary preparation techniques using in the SEM imaging of biofilms, SEM still revealed differences between Tob-PEG and tobramycin treated biofilms. Similar to the confocal images, control *P. aeruginosa* biofilms exhibited dense colonization with a clearly visible extracellular matrix. These biofilms showed highly organized and well defined architecture (Fig. 6A). PEG treated biofilms also exhibited a similar dense and well organized extracellular matrix further confirming that PEG itself did not significantly affect the overall structure of *P. aeruginosa* biofilms (Fig. 6B). Both tobramycin and Tob-PEG treated biofilms at their respective MIC_{80}s demonstrated significant disruption of the biofilm structure. However, tobramycin treated biofilms showed some evidence of organization throughout the remaining bacterial cells with some quantities of extracellular matrix visible (Fig. 6C). In contrast, only a few scattered bacterial cells were noted in Tob-PEG treated biofilms (Fig. 6D). This showed that the chemical conjugation of PEG to tobramycin had a significant negative effect on the *P. aeruginosa* biofilm structure and matrix when compared to the biofilms treated with both agents individually.

6.5 DISSCUSION

As introduced above, biofilms are well known to be extremely resistant to antimicrobials. Hence, the principal objective of most novel anti-biofilm strategies is to develop antimicrobials that are more efficient at killing biofilms under lower therapeutic doses. To achieve that objective, we have tested aforementioned compounds on established *P. aeruginosa* biofilms.

6.5.1 Tob-PEG Exerted a Superior Antibiofilm Effect on the *P. aeruginosa* Biofilms When Compared to Tobramycin

Tob-PEG showed greater anti-biofilm properties over conventional tobramycin towards biofilms. The results obtained from the biofilms phase confirmed these findings. Despite lower activity in the planktonic phase as indicated by a higher MIC_{80}, Tob-PEG had significantly improved (over 3 fold) activity the in the biofilms with low MIC_{80} (Fig. 3). To rule out the possibility of the increased inhibitory properties of tobramycin due to physical presence of PEG in the formulation, a mixture of both tobramycin and PEG was also investigated. This physical mixture failed to elicit similar significant effect as chemically conjugated tobramycin and PEG. These data confirmed that the process of chemical conjugation and the final structure of the novel molecule was an important factor for the antibiofilm activity.

It is well-known that biofilms exhibit resistance to antimicrobials via nonspecifically binding to biofilm matrix (38). This slow penetration results in cascade of phenotypic changes, which allows the adjacent bacteria to trigger stress responses and become resistant to the specific agent administered. For tobramycin, the higher MIC_{80} observed in the biofilms is likely ascribed to the ionic interactions between tobramycin

and biofilm matrix. This interaction limits the penetration of tobramycin through the alginate matrix in *P. aeruginosa* biofilms and impacts its therapeutic efficacy (38). In contrast, the PEG moiety in the Tob-PEG molecule has a relatively large molecular weight (5000 Da) and appears to form a molecular shield to cover the cationic charges in tobramycin. As previously reported, PEGylation of particles enables enhanced penetration of substances through charged matrices such as mucins (45). Therefore we hypothesize that tobramycin is converted to a new therapeutic form, with less binding affinity to biofilm matrix, upon the covalent addition of a PEG moiety. This allows for greater penetration into the interior of the biofilm and therefore lowers MICs.

MICs in the planktonic phase of microorganisms are typically reported as MIC_{90} or MIC_{100}. In biofilms, however, resistance to antibiotic treatments results in very high drug concentrations required to achieve this 90% or above death of the bacteria (16, 58). Therefore, in most experimental cases, if not all, achieving these high concentrations is unpractical and extremely difficult due to chemical and physical properties of the specific drug (ie. solubility limits, tonicity effects, etc.). Therefore, unlike their planktonic counterparts, there are no established standards such as Clinical and Laboratory Standards Institute (CLSI) guidelines for measuring MICs of biofilms. MIC_{80} values of antimicrobial agents had been increasingly used in recent studies focusing on biofilms as an acceptable parameter (59, 60). Accordingly, MIC_{80} was used in the current study as a standardized measure of antibacterial efficacy for comparisons between the different treatments.

Consistent with the antimicrobial activity, the microscopy studies presented here also displayed a significant difference between tobramycin and Tob-PEG treatment in the

P. aeruginosa biofilms. Both live and dead staining, confocal microscopy, and SEM images confirmed that tobramycin and Tob-PEG could severely disrupt the biofilm architecture. However, the destruction resultant from the Tob-PEG treatment was more severe than that of only tobramycin, as the biofilms treated with tobramycin showed a certain degree of preservation of the three-dimensional architecture and extracellular matrix (Fig. 6C). The microscopic images also confirmed that PEG itself did not significantly affect *P. aeruginosa* biofilms.

6.5.2 Tob-PEG Did Not Benefit the Elimination of Planktonic *P. aeruginosa*

Aminoglycosides, the family of antibiotics that tobramycin belongs to, are hydrophilic sugars with amino and hydroxyl functional groups (61). The amine moieties of aminoglycosides become protonated in physiological conditions making aminoglycosides polycationic. Due to this polycationic nature, aminoglycosides like tobramycin exhibit a greater affinity of binding to bacterial nucleic acids. Particularly, aminoglycosides possess a high affinity to certain portions of RNAs, such as prokaryotic 16S rRNA (61). The latter, a small ribosomal subunit, has been identified as the primary target of aminoglycosides. Aminoglycoside binding to 16S rRNA impairs "translational proofreading" giving rise to misreading or premature termination of the RNA message, or both. Subsequently, an inaccurately translated protein product is resulted. This subset of abnormal proteins can subsequently be incorporated into the cell membrane of a bacterium, leading to altered permeability and up regulation of amino acid influx. This cycle continues until cell death occurs (32, 62).

When tested in the planktonic phase, Tob-PEG showed approximately 10 fold reduction of its efficacy in achieving MIC_{80} compared to conventional tobramycin (Fig.

3). This is not an unexpected finding given that the 5000 kDa PEG attached to the tobramycin is likely to influence antibacterial activity via several mechanisms. Firstly, the conjugation of PEG to the tobramycin molecule can diminish the binding efficiency of tobramycin to 16S rRNA by the steric hindrance introduced by the addition of a PEG compared to the non-conjugated tobramycin. Secondly, the conjugation of PEG to the 6' amine group of tobramycin yielded a molecule, Tob-PEG, with a decreased cationic nature, as shown in Figure 4, thereby decreasing electronic interactions of the drug and the target ribosome. Thirdly, the process of cellular uptake of tobramycin into the bacteria has been reported to be self-promoted, which involves the aminoglycoside-induced disruption of Mg^{2+} bridges between adjacent lipopolysaccharide molecules in the outer membrane of the bacteria (63, 64). Thus, the conjugation of PEG to tobramycin may significantly affect this uptake mechanism and consequently decrease its cellular uptake. These potential reasons for the lower antimicrobial activity of Tob-PEG in the planktonic phase of *P. aeruginosa* will be explored further. Moreover, it is important to note that PEG alone, as a control, did not cause any significant changes to *P. aeruginosa* growth in the planktonic phase indicating that PEG itself was neither an inhibitor nor promoter of microbial activity (65).

6.5.3 Visual Alginate and Drug Interaction Study

It has been previously reported tobramycin may bind to alginate in the bacterial biofilm through ionic interactions (38). This binding has been proposed to limit the permeation of tobramycin into the biofilm and subsequently result in lower drug concentrations reaching the bacterial cell target site (66). This has been suggested as one potential reason for bacterial biofilm antibiotic resistance. In this study, our working

hypothesis was that the conjugation of PEG to tobramycin would diminish the positive charge of tobramycin and consequently diminish the ionic interactions between alginate and tobramycin thereby facilitating the permeation of tobramycin into the bacterial biofilm. Beyond the antibacterial performance studies, we performed studies that illustrated the clear difference in drug interactions with alginate using the PEG conjugate. As shown in Figure 4C, no visible interaction with alginate was observed after tobramycin was chemically modified into Tob-PEG in contrast to the unconjugated drug (Fig. 4B). The absence of interaction can be attributed to the PEG conjugated at the tobramycin 6' amine site, which reduces the overall positive charge of tobramycin. In addition, the polymeric PEG, which has a molecular weight of 5000 kDa, is proposed to form a hydration shield around the tobramycin, which would also decrease the likelihood of physical interactions between the tobramycin and alginate. This is illustrated by the differences in the interactions observed in Figure 4C and 4D. In Figure 4D, the droplet of the added mixture of tobramycin and PEG dispersed more easily into the alginate than was observed with tobramycin alone. This indicated that PEG, even unbound to the tobramycin could achieve some level of shielding, though significant interactions were still observed.

6.6 CONCLUSION

To overcome biofilm-associated antibacterial resistance, a new compound of tobramycin-PEG (Tob-PEG) was developed via conjugating tobramycin to polyethylene glycol (PEG). Compared to tobramycin, Tob-PEG exhibited almost 10 fold less inhibition efficiency on *P. aeruginosa* in its planktonic phase. However, Tob-PEG suppressed *P. aeruginosa* growth in its biofilm phase with much lower concentration than

tobramycin (27.8 µmol/L vs. 89.8 µmol/L). The mixed PEG and tobramycin (ie. non-conjugated) behaved as same as tobramycin, indicating that the covalently bound complex Tob-PEG does have unique qualities. The novel compound presented and characterized here, Tob-PEG, provides a promising direction for overcoming bacterial resistance due to biofilm formation, a key to developing further therapies for congenital conditions such as CF. Additional investigations are currently underway to further understand the mechanisms for improved efficacy of this compound., as well as the combination usage of tobramycin and Tob-PEG on *P. aeruginosa* biofilms.

6.7 ACKNOWLEDGEMENTS

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6.8 FIGURES

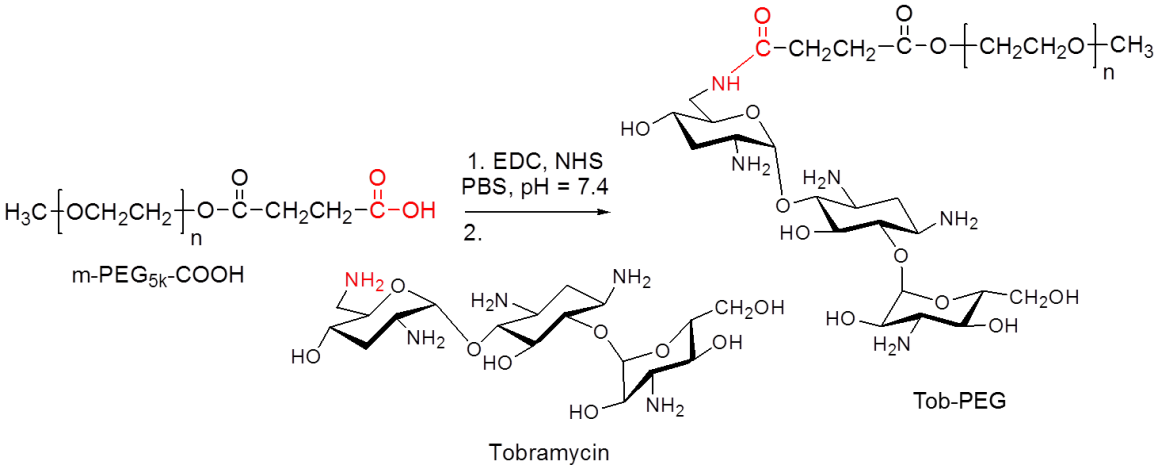

Figure 6.1 Schematic illustration of synthesis of polyethylene glycol conjugated tobramycin (Tob-PEG).

Abbreviations: PEG, polyethylene glycol; Tob-PEG, polyethylene glycol conjugated tobramycin; EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, N-Hydroxysuccinimide; PBS, phosphate buffered saline.

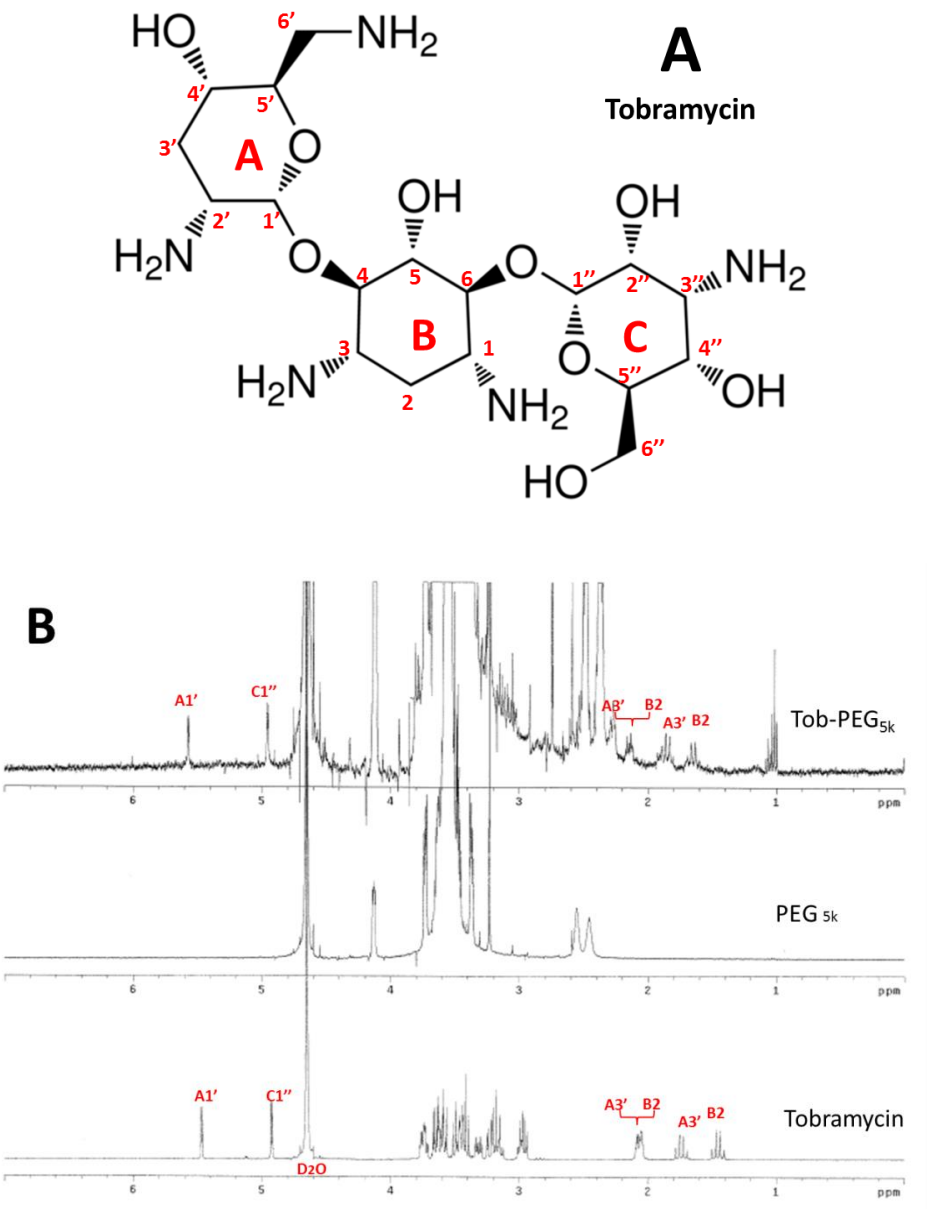

Figure 6.2 H-NMR spectrum of tobramycin, PEG_{5K}, and Tob-PEG_{5k} in D_2O. A, chemical structure of tobramycin with marked H atoms at different locations; B, H-NMR spectrum of tobramycin, PEG_{5K} and Tob-PEG_{5k}.

Abbreviations: PEG, polyethylene glycol; Tob-PEG, polyethylene glycol conjugated tobramycin.

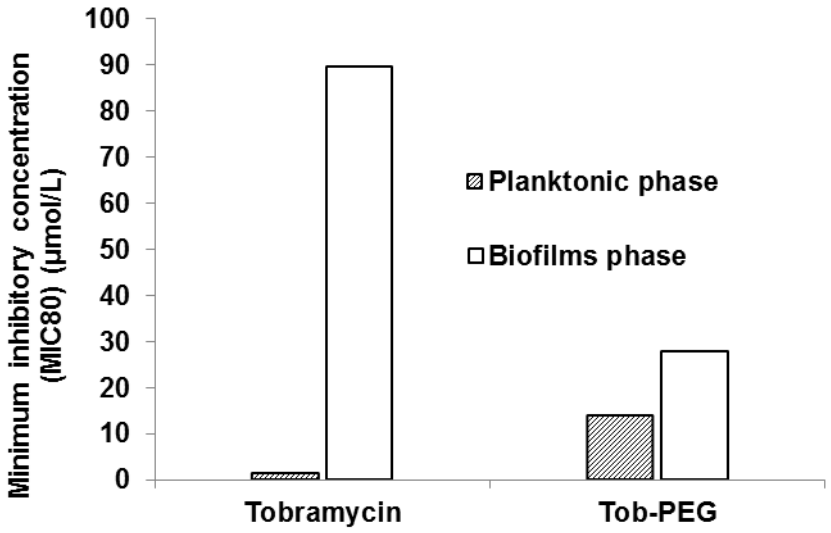

Figure 6.3 Minimum inhibitory concentration (MIC_{80}) of tobramycin and Tob-PEG in planktonic phase and biofilm phage of *P. aeruginosa.* (MIC_{80}±SD, SD=0, n=12, Experiments were performed in quadruplicates three times. The broth dilution assay resulted in the same value of the drug concentration for MIC_{80}, thus SD was 0.)

Abbreviation: Tob-PEG, polyethylene glycol conjugated tobramycin.

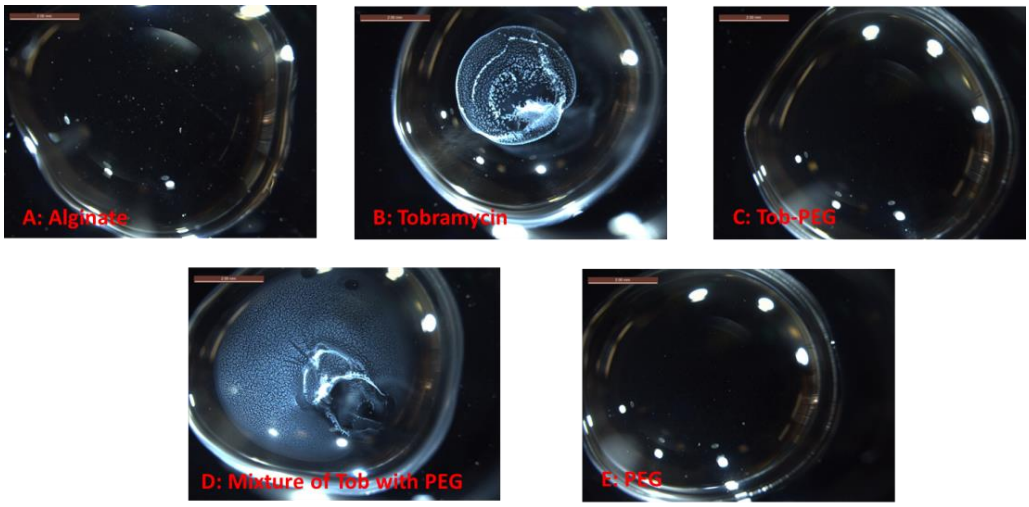

Figure 6.4 Visual alginate and drug interaction study. A, alginate solution droplet; B, interaction between alginate and tobramycin; C, interaction between alginate and Tob-PEG; D, interaction between alginate and the mixture of tobramycin and PEG; E, interaction between alginate and PEG. Bar: 2.0 mm.

Abbreviations: Tob, tobramycin; PEG, polyethylene glycol; Tob-PEG, polyethylene glycol conjugated tobramycin.

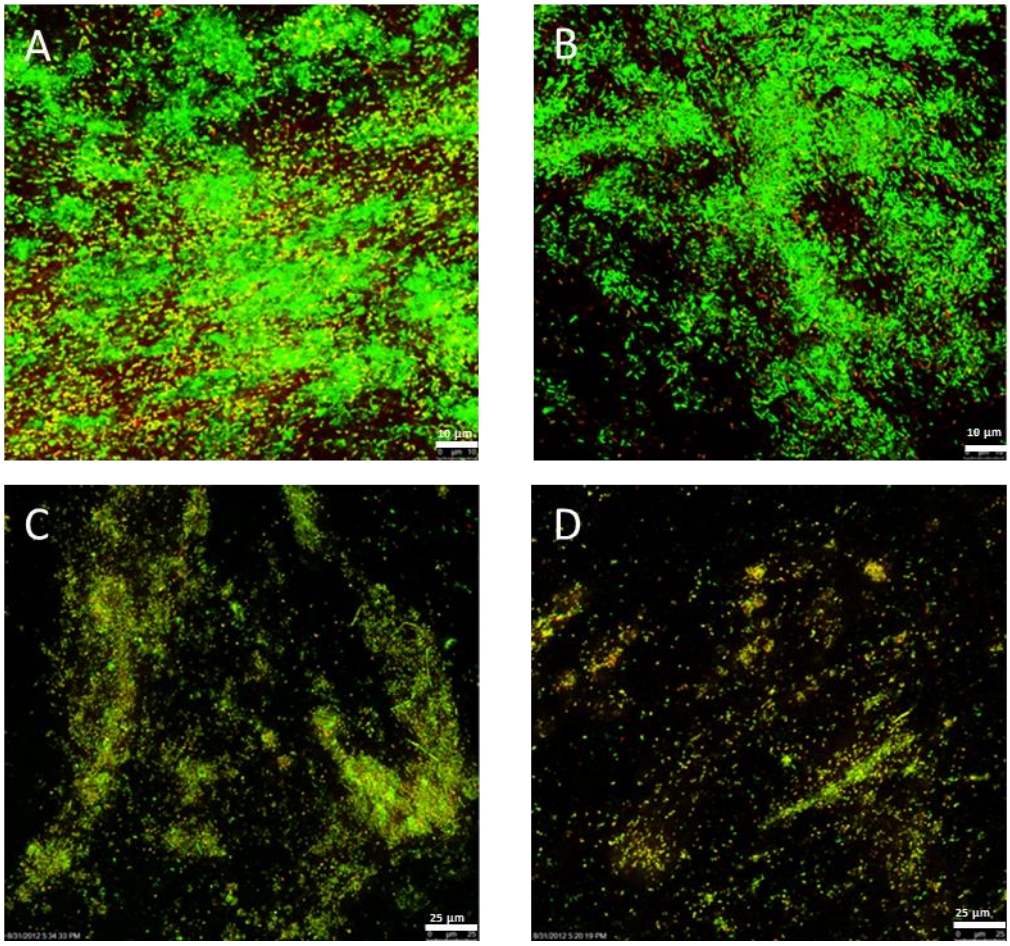

Figure 6.5 Confocal images of *P. aeruginosa* biofilm. Stained with Live/Dead BacLight Bacterial Viability kit. Live cells were stained in green and dead cells stained in red. A, control *P. aeruginosa* biofilms; B, biofilms treated with PEG; C, biofilms treated with tobramycin; D, biofilms treated with Tob-PEG.

Abbreviation: PEG, polyethylene glycol; Tob-PEG, polyethylene glycol conjugated tobramycin.

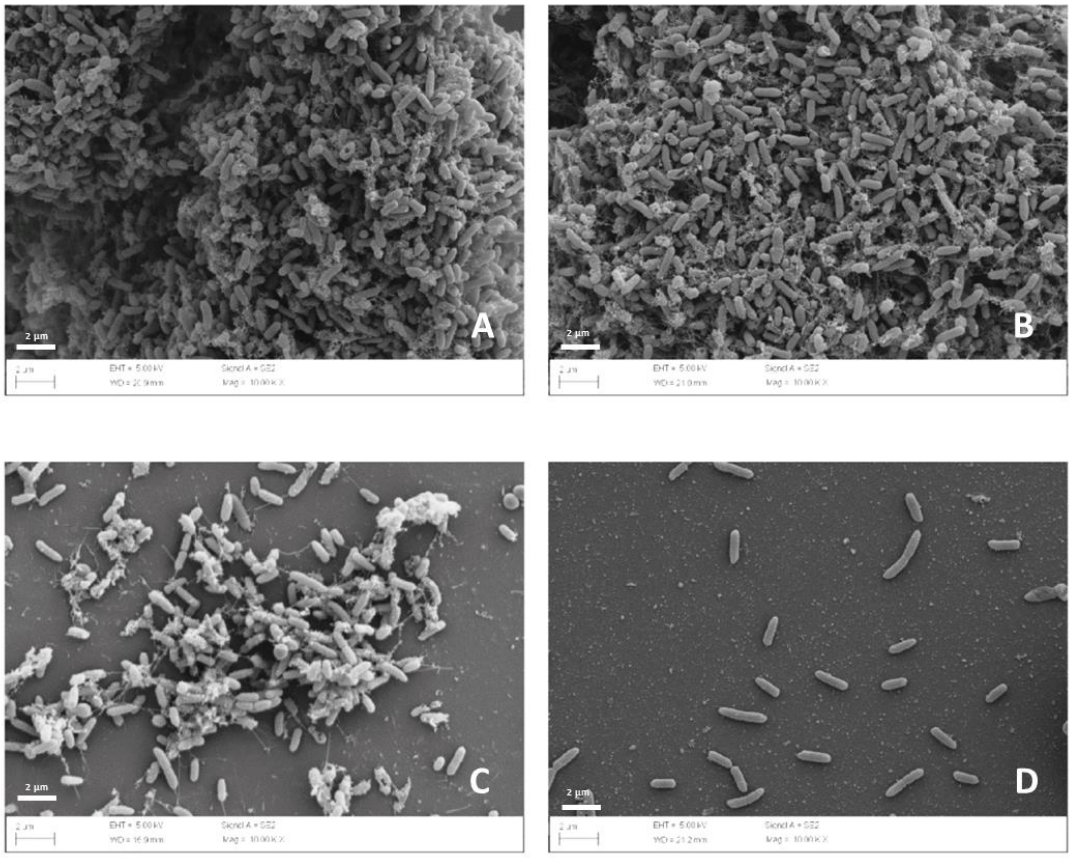

Figure 6.6 SEM images of *P. aeruginosa* biofilm (×10000). A, control *P. aeruginosa* biofilms; B, biofilms treated with PEG; C, biofilms treated with tobramycin; D, biofilms treated with Tob-PEG.

Abbreviations: PEG, polyethylene glycol; Tob-PEG, polyethylene glycol conjugated tobramycin.

6.9 REFERENCES

1. Boucher RC. New concepts of the pathogenesis of cystic fibrosis lung disease. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2004;23(1):146-58. Epub 2004/01/24.

2. Hoiby N, Krogh Johansen H, Moser C, Song Z, Ciofu O, Kharazmi A. *Pseudomonas aeruginosa* and the *in vitro* and *in vivo* biofilm mode of growth. Microbes and infection / Institut Pasteur. 2001;3(1):23-35. Epub 2001/02/28.

3. Moss RB. Infection, inflammation, and the downward spiral of cystic fibrosis lung disease. The Journal of pediatrics. 2009;154(2):162-3.

4. Frederiksen B, Koch C, Hoiby N. Changing epidemiology of *Pseudomonas aeruginosa* infection in Danish cystic fibrosis patients (1974-1995). Pediatric pulmonology. 1999;28(3):159-66. Epub 1999/09/24.

5. Szaff M, Hoiby N, Flensborg EW. Frequent antibiotic therapy improves survival of cystic fibrosis patients with chronic *Pseudomonas aeruginosa* infection. Acta paediatrica Scandinavica. 1983;72(5):651-7. Epub 1983/09/01.

6. Hoffmann N, Rasmussen TB, Jensen PO, Stub C, Hentzer M, Molin S, et al. Novel mouse model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis. Infection and immunity. 2005;73(4):2504-14. Epub 2005/03/24.

7. Jelsbak L, Johansen HK, Frost AL, Thogersen R, Thomsen LE, Ciofu O, et al. Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis patients. Infection and immunity. 2007;75(5):2214-24. Epub 2007/01/31.

8. Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatric pulmonology. 2009;44(6):547-58. Epub 2009/05/07.

9. Samaranayake LP. Essential Microbiology for Dentistry. Edinburgh: Churchill Livingstone; 2006.

10. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001;9(1):34-9. Epub 2001/02/13.

11. Potera C. Antibiotic Resistance: Biofilm Dispersing Agent Rejuvenates Older Antibiotics. Environmental Health Perspectives. 2010;118(7):A288.

12. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents. 2010;35(4):322-32. Epub 2010/02/13.

13. d'Angelo I, Conte C, La Rotonda MI, Miro A, Quaglia F, Ungaro F. Improving the efficacy of inhaled drugs in cystic fibrosis: Challenges and emerging drug delivery strategies. Adv Drug Deliv Rev. 2014;75C:92-111. Epub 2014/05/21.

14. Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. Trends Microbiol. 2005;13(1):34-40. Epub 2005/01/11.

15. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet. 2001;358(9276):135-8. Epub 2001/07/21.

16. Hoiby N, Ciofu O, Bjarnsholt T. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. Future microbiology. 2010;5(11):1663-74. Epub 2010/12/08.

17. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284(5418):1318-22. Epub 1999/05/21.

18. Lee B, Haagensen JA, Ciofu O, Andersen JB, Hoiby N, Molin S. Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. J Clin Microbiol. 2005;43(10):5247-55. Epub 2005/10/07.

19. George AM, Jones PM, Middleton PG. Cystic fibrosis infections: treatment strategies and prospects. FEMS Microbiol Lett. 2009;300(2):153-64. Epub 2009/08/14.

20. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010;8(9):623-33. Epub 2010/08/03.

21. Giwercman B, Lambert PA, Rosdahl VT, Shand GH, Hoiby N. Rapid emergence of resistance in *Pseudomonas aeruginosa* in cystic fibrosis patients due to *in-vivo* selection of stable partially derepressed beta-lactamase producing strains. The Journal of antimicrobial chemotherapy. 1990;26(2):247-59. Epub 1990/08/01.

22. Giwercman B, Meyer C, Lambert PA, Reinert C, Hoiby N. High-level beta-lactamase activity in sputum samples from cystic fibrosis patients during antipseudomonal treatment. Antimicrobial agents and chemotherapy. 1992;36(1):71-6. Epub 1992/01/01.

23. Jalal S, Ciofu O, Hoiby N, Gotoh N, Wretlind B. Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrobial agents and chemotherapy. 2000;44(3):710-2. Epub 2000/02/19.

24. Saiman L, Mehar F, Niu WW, Neu HC, Shaw KJ, Miller G, et al. Antibiotic susceptibility of multiply resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis, including candidates for transplantation. Clinical infectious diseases : an

official publication of the Infectious Diseases Society of America. 1996;23(3):532-7. Epub 1996/09/01.

25. Westbrock-Wadman S, Sherman DR, Hickey MJ, Coulter SN, Zhu YQ, Warrener P, et al. Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. Antimicrobial agents and chemotherapy. 1999;43(12):2975-83. Epub 1999/12/03.

26. Burns JL, Van Dalfsen JM, Shawar RM, Otto KL, Garber RL, Quan JM, et al. Effect of chronic intermittent administration of inhaled tobramycin on respiratory microbial flora in patients with cystic fibrosis. The Journal of infectious diseases. 1999;179(5):1190-6. Epub 1999/04/07.

27. MacLeod DL, Nelson LE, Shawar RM, Lin BB, Lockwood LG, Dirk JE, et al. Aminoglycoside-resistance mechanisms for cystic fibrosis *Pseudomonas aeruginosa* isolates are unchanged by long-term, intermittent, inhaled tobramycin treatment. The Journal of infectious diseases. 2000;181(3):1180-4. Epub 2000/03/18.

28. Denton M, Kerr K, Mooney L, Keer V, Rajgopal A, Brownlee K, et al. Transmission of colistin-resistant *Pseudomonas aeruginosa* between patients attending a pediatric cystic fibrosis center. Pediatric pulmonology. 2002;34(4):257-61. Epub 2002/09/03.

29. Johansen HK, Moskowitz SM, Ciofu O, Pressler T, Hoiby N. Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2008;7(5):391-7. Epub 2008/03/25.

30. Anderson GG, Kenney TF, Macleod DL, Henig NR, O'Toole GA. Eradication of *Pseudomonas aeruginosa* biofilms on cultured airway cells by a fosfomycin/tobramycin antibiotic combination. Pathog Dis. 2013;67(1):39-45. Epub 2013/04/27.

31. Mesaros N, Nordmann P, Plesiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, et al. *Pseudomonas aeruginosa:* resistance and therapeutic options at the turn of the new millennium. Clin Microbiol Infect. 2007;13(6):560-78. Epub 2007/02/03.

32. Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. Aminoglycosides: activity and resistance. Antimicrobial agents and chemotherapy. 1999;43(4):727-37. Epub 1999/04/02.

33. Tre-Hardy M, Traore H, El Manssouri N, Vanderbist F, Vaneechoutte M, Devleeschouwer MJ. Evaluation of long-term co-administration of tobramycin and clarithromycin in a mature biofilm model of cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. Int J Antimicrob Agents. 2009;34(4):370-4. Epub 2009/06/10.

34. Woodward TC, Brown R, Sacco P, Zhang J. Budget impact model of tobramycin inhalation solution for treatment of *Pseudomonas aeruginosa* in cystic fibrosis patients. J Med Econ. 2010;13(3):492-9. Epub 2010/07/31.

35. McKeage K. Tobramycin inhalation powder: a review of its use in the treatment of chronic *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. Drugs. 2013;73(16):1815-27. Epub 2013/11/07.

36. Pier GB. The challenges and promises of new therapies for cystic fibrosis. J Exp Med. 2012;209(7):1235-9. Epub 2012/07/04.

37. Ratjen FA. Cystic fibrosis: pathogenesis and future treatment strategies. Respir Care. 2009;54(5):595-605. Epub 2009/04/28.

38. Tseng BS, Zhang W, Harrison JJ, Quach TP, Song JL, Penterman J, et al. The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. Environ Microbiol. 2013;15(10):2865-78. Epub 2013/06/12.

39. Stewart PS. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob Agents Chemother. 1996;40(11):2517-22. Epub 1996/11/01.

40. Alconcel SNS, Baas AS, Maynard HD. FDA-approved poly(ethylene glycol)–protein conjugate drugs. Polymer Chemistry. 2011;2:1442-8.

41. Veronese FM, Schiavon O, Pasut G, Mendichi R, Andersson L, Tsirk A, et al. PEG-doxorubicin conjugates: influence of polymer structure on drug release, *in vitro* cytotoxicity, biodistribution, and antitumor activity. Bioconjug Chem. 2005;16(4):775-84. Epub 2005/07/21.

42. Yang H, Lopina ST. Penicillin V-conjugated PEG-PAMAM star polymers. J Biomater Sci Polym Ed. 2003;14(10):1043-56. Epub 2003/12/10.

43. Liu Z, Robinson JT, Sun X, Dai H. PEGylated nanographene oxide for delivery of water-insoluble cancer drugs. Journal of the American Chemical Society. 2008;130(33):10876-7.

44. van Vlerken LE, Vyas TK, Amiji MM. Poly (ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. Pharmaceutical research. 2007;24(8):1405-14.

45. Suk JS, Kim AJ, Trehan K, Schneider CS, Cebotaru L, Woodward OM, et al. Lung gene therapy with highly compacted DNA nanoparticles that overcome the mucus barrier. Journal of controlled release : official journal of the Controlled Release Society. 2014;178:8-17. Epub 2014/01/21.

46. Shaul P, Green KD, Rutenberg R, Kramer M, Berkov-Zrihen Y, Breiner-Goldstein E, et al. Assessment of 6'- and 6'"-N-acylation of aminoglycosides as a strategy to overcome bacterial resistance. Org Biomol Chem. 2011;9(11):4057-63. Epub 2011/03/03.

47. Luten J, van Steenbergen MJ, Lok MC, de Graaff AM, van Nostrum CF, Talsma H, et al. Degradable PEG-folate coated poly(DMAEA-*co*-BA)phosphazene-based polyplexes exhibit receptor-specific gene expression. Eur J Pharm Sci. 2008;33(3):241-51. Epub 2008/01/22.

48. Popielarski SR, Pun SH, Davis ME. A nanoparticle-based model delivery system to guide the rational design of gene delivery to the liver. 1. Synthesis and characterization. Bioconjug Chem. 2005;16(5):1063-70. Epub 2005/09/22.

49. Wu Y, Liu C, Zhao X, Xiang J. A new biodegradable polymer: PEGylated chitosan-g-PEI possessing a hydroxyl group at the PEG end. Journal of Polymer Research. 2008;15(3):181-5.

50. El-Sherbiny IM, McGill S, Smyth HD. Swellable microparticles as carriers for sustained pulmonary drug delivery. J Pharm Sci. 2010;99(5):2343-56. Epub 2009/12/08.

51. Bandara HM, Yau JY, Watt RM, Jin LJ, Samaranayake LP. *Pseudomonas aeruginosa* inhibits *in-vitro Candida* biofilm development. BMC Microbiol. 2010;10:125. Epub 2010/04/27.

52. Bandara HM, Cheung BPK, Watt RM, Jin LJ, Samaranayake LP. *Pseudomonas aeruginosa* lipopolysaccharide inhibits *Candida albicans* hyphae formation and alters gene expression during biofilm development. Mol Oral Microbiol. 2013;28(1):54-69. Epub 2012/12/01.

53. Bandara HM, Lam OL, Watt RM, Jin LJ, Samaranayake LP. Bacterial lipopolysaccharides variably modulate *in vitro* biofilm formation of *Candida* species. J Med Microbiol. 2010;59(Pt 10):1225-34. Epub 2010/06/26.

54. Alhede M, Qvortrup K, Liebrechts R, Hoiby N, Givskov M, Bjarnsholt T. Combination of microscopic techniques reveals a comprehensive visual impression of biofilm structure and composition. FEMS Immunol Med Microbiol. 2012;65(2):335-42. Epub 2012/03/21.

55. Reznikov LR, Abou Alaiwa MH, Dohrn CL, Gansemer ND, Diekema DJ, Stoltz DA, et al. Antibacterial properties of the CFTR potentiator ivacaftor. J Cyst Fibros. 2014;13(5):515-9. Epub 2014/03/13.

56. Tin S, Sakharkar KR, Lim CS, Sakharkar MK. Activity of Chitosans in combination with antibiotics in *Pseudomonas aeruginosa*. Int J Biol Sci. 2009;5(2):153-60. Epub 2009/01/29.

57. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163-75. Epub 2008/02/16.

58. Nickel JC, Ruseska I, Wright JB, Costerton JW. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob Agents Chemother. 1985;27(4):619-24. Epub 1985/04/01.

59. Klepser ME, Ernst EJ, Ernst ME, Messer SA, Pfaller MA. Evaluation of endpoints for antifungal susceptibility determinations with LY303366. Antimicrob Agents Chemother. 1998;42(6):1387-91. Epub 1998/06/13.

60. Xu X, Zhou XD, Wu CD. Tea catechin epigallocatechin gallate inhibits *Streptococcus mutans* biofilm formation by suppressing gtf genes. Arch Oral Biol. 2012;57(6):678-83. Epub 2011/12/16.

61. Kotra LP, Haddad J, Mobashery S. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob Agents Chemother. 2000;44(12):3249-56. Epub 2000/11/18.

62. Magnet S, Blanchard JS. Molecular insights into aminoglycoside action and resistance. Chem Rev. 2005;105(2):477-98. Epub 2005/02/11.

63. Hancock RE, Farmer SW, Li ZS, Poole K. Interaction of aminoglycosides with the outer membranes and purified lipopolysaccharide and OmpF porin of *Escherichia coli*. Antimicrob Agents Chemother. 1991;35(7):1309-14. Epub 1991/07/01.

64. Jana S, Deb JK. Molecular understanding of aminoglycoside action and resistance. Appl Microbiol Biotechnol. 2006;70(2):140-50. Epub 2006/01/05.

65. Wu L, Zaborina O, Zaborin A, Chang EB, Musch M, Holbrook C, et al. High-molecular-weight polyethylene glycol prevents lethal sepsis due to intestinal *Pseudomonas aeruginosa*. Gastroenterology. 2004;126(2):488-98. Epub 2004/02/06.

66. Dhondikubeer R, Bera S, Zhanel GG, Schweizer F. Antibacterial activity of amphiphilic tobramycin. The Journal of antibiotics. 2012;65(10):495-8.

Bibliography

Adi, H., P. M. Young, et al. (2010). "Controlled release antibiotics for dry powder lung delivery." Drug Dev Ind Pharm **36**(1): 119-126.

Ahsan, F., I. P. Rivas, et al. (2002). "Targeting to macrophages: role of physicochemical properties of particulate carriers--liposomes and microspheres--on the phagocytosis by macrophages." J Control Release **79**(1-3): 29-40.

Akala, E. O., P. Kopeckova, et al. (1998). "Novel pH-sensitive hydrogels with adjustable swelling kinetics." Biomaterials **19**(11-12): 1037-1047.

Akinloye, O. M., E. Ronkko, et al. (2011). "Specific viruses detected in nigerian children in association with acute respiratory disease." J Trop Med **2011**: 690286.

Alconcel, S. N. S., A. S. Baas, et al. (2011). "FDA-approved poly(ethylene glycol)–protein conjugate drugs." Polymer Chemistry **2**: 1442-1448.

Alhede, M., K. Qvortrup, et al. (2012). "Combination of microscopic techniques reveals a comprehensive visual impression of biofilm structure and composition." FEMS Immunol Med Microbiol **65**(2): 335-342.

Allcorn, E. and A. Manthiram (2014). "NiSb–Al2O3–C Nanocomposite Anodes with Long Cycle Life for Li-Ion Batteries." The Journal of Physical Chemistry C **118**(2): 811-822.

Amidi, M., E. Mastrobattista, et al. (2010). "Chitosan-based delivery systems for protein therapeutics and antigens." Adv Drug Deliv Rev **62**(1): 59-82.

Amidi, M., H. C. Pellikaan, et al. (2007). "Diphtheria toxoid-containing microparticulate powder formulations for pulmonary vaccination: preparation, characterization and evaluation in guinea pigs." Vaccine **25**(37): 6818-6829.

Amin, R. and F. Ratjen (2008). "Cystic fibrosis: a review of pulmonary and nutritional therapies." Adv Pediatr **55**: 99-121.

Anderson, G. G., T. F. Kenney, et al. (2013). "Eradication of *Pseudomonas aeruginosa* biofilms on cultured airway cells by a fosfomycin/tobramycin antibiotic combination." Pathog Dis **67**(1): 39-45.

Aristilde, L. and G. Sposito (2013). "Complexes of the antimicrobial ciprofloxacin with soil, peat, and aquatic humic substances." Environmental Toxicology and Chemistry **32**(7): 1467-1478.

Arnold, M. M., E. M. Gorman, et al. (2007). "NanoCipro encapsulation in monodisperse large porous PLGA microparticles." J Control Release **121**(1-2): 100-109.

Arora, D., K. A. Shah, et al. (2010). "In vitro aqueous fluid-capacity-limited dissolution testing of respirable aerosol drug particles generated from inhaler products." Pharmaceutical research **27**(5): 786-795.

Arya, V., I. Coowanitwong, et al. (2006). "Pulmonary targeting of sustained release formulation of budesonide in neonatal rats." J Drug Target **14**(10): 680-686.

Augst, A. D., H. J. Kong, et al. (2006). "Alginate hydrogels as biomaterials." Macromolecular bioscience **6**(8): 623-633.

Baier Leach, J., K. A. Bivens, et al. (2003). "Photocrosslinked hyaluronic acid hydrogels: natural, biodegradable tissue engineering scaffolds." Biotechnol Bioeng **82**(5): 578-589.

Bailey, M. M. and C. J. Berkland (2009). "Nanoparticle formulations in pulmonary drug delivery." Medicinal research reviews **29**(1): 196-212.

Balashazy, I., W. Hofmann, et al. (2008). "Three-dimensional model for aerosol transport and deposition in expanding and contracting alveoli." Inhal Toxicol **20**(6): 611-621.

Balasubramanian, S. K., K. W. Poh, et al. (2013). "The effect of primary particle size on biodistribution of inhaled gold nano-agglomerates." Biomaterials **34**(22): 5439-5452.

Bandara, H. M., B. P. K. Cheung, et al. (2013). "*Pseudomonas aeruginosa* lipopolysaccharide inhibits *Candida albicans* hyphae formation and alters gene expression during biofilm development." Mol Oral Microbiol **28**(1): 54-69.
Bandara, H. M., O. L. Lam, et al. (2010). "Bacterial lipopolysaccharides variably modulate *in vitro* biofilm formation of *Candida* species." J Med Microbiol **59**(Pt 10): 1225-1234.
Bandara, H. M., J. Y. Yau, et al. (2010). "*Pseudomonas aeruginosa* inhibits *in-vitro Candida* biofilm development." BMC Microbiol **10**: 125.
Barnes, P. J. (2006). "How corticosteroids control inflammation: Quintiles Prize Lecture 2005." Br J Pharmacol **148**(3): 245-254.
Beck-Broichsitter, M., J. Gauss, et al. (2009). "Pulmonary drug delivery with aerosolizable nanoparticles in an ex vivo lung model." Int J Pharm **367**(1-2): 169-178.
Beck-Broichsitter, M., M. Rieger, et al. (2012). "Correlation of drug release with pulmonary drug absorption profiles for nebulizable liposomal formulations." Eur J Pharm Biopharm.
Beck-Broichsitter, M., C. Schweiger, et al. (2012). "Characterization of novel spray-dried polymeric particles for controlled pulmonary drug delivery." J Control Release **158**(2): 329-335.
Bhattarai, N., J. Gunn, et al. (2010). "Chitosan-based hydrogels for controlled, localized drug delivery." Advanced drug delivery reviews **62**(1): 83-99.
Bi, R., W. Shao, et al. (2008). "Spray-freeze-dried dry powder inhalation of insulin-loaded liposomes for enhanced pulmonary delivery." J Drug Target **16**(9): 639-648.
Biswas, S., P. P. Deshpande, et al. (2013). "Octa-arginine-modified pegylated liposomal doxorubicin: An effective treatment strategy for non-small cell lung cancer." Cancer Lett.
Bivas-Benita, M., M. Y. Lin, et al. (2009). "Pulmonary delivery of DNA encoding Mycobacterium tuberculosis latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice." Vaccine **27**(30): 4010-4017.
Bivas-Benita, M., S. Romeijn, et al. (2004). "PLGA-PEI nanoparticles for gene delivery to pulmonary epithelium." Eur J Pharm Biopharm **58**(1): 1-6.
Bjarnsholt, T., P. O. Jensen, et al. (2009). "*Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients." Pediatr Pulmonol **44**(6): 547-558.
Bo Olsson, E. B., Lars Borgstrom, Staffan Edsbacker, Stefan Eirefelt, Kararina Ekelund, Lena Gustavsson, Tova Hegelund-Myrback (2011). Controlled Pulmonary Drug Delivery. New York, Spring Science+Business Media.
Boucher, R. C. (2004). "New concepts of the pathogenesis of cystic fibrosis lung disease." Eur Respir J **23**(1): 146-158.
Boucher, R. C., M. J. Stutts, et al. (1981). "Regional differences in bioelectric properties and ion flow in excised canine airways." J Appl Physiol **51**(3): 706-714.
Boura, C., P. Menu, et al. (2003). "Endothelial cells grown on thin polyelectrolyte mutlilayered films: an evaluation of a new versatile surface modification." Biomaterials **24**(20): 3521-3530.
Boura, C., S. Muller, et al. (2005). "Endothelial cell--interactions with polyelectrolyte multilayer films." Biomaterials **26**(22): 4568-4575.
Brannon-Peppas, L. (1997). "Med. Plastics. Biomater." Med. Plastics. Biomater **4**: 34-44.
Bur, M., H. Huwer, et al. (2006). "Assessment of transport rates of proteins and peptides across primary human alveolar epithelial cell monolayers." Eur J Pharm Sci **28**(3): 196-203.
Burns, J. L., J. M. Van Dalfsen, et al. (1999). "Effect of chronic intermittent administration of inhaled tobramycin on respiratory microbial flora in patients with cystic fibrosis." J Infect Dis **179**(5): 1190-1196.
Byron, P. R. (1986). "Prediction of drug residence times in regions of the human respiratory tract following aerosol inhalation." Journal of pharmaceutical sciences **75**(5): 433-438.

Cazzola, M., R. Testi, et al. (2002). "Clinical pharmacokinetics of salmeterol." Clin Pharmacokinet **41**(1): 19-30.
Champion, J. A., A. Walker, et al. (2008). "Role of particle size in phagocytosis of polymeric microspheres." Pharm Res **25**(8): 1815-1821.
Chan, G. and D. J. Mooney (2013). "Ca< sup> 2+</sup> released from calcium alginate gels can promote inflammatory responses< i> in vitro</i> and< i> in vivo</i>." Acta biomaterialia **9**(12): 9281-9291.
Chen, K., Q. Zhang, et al. (2011). "Effect of Cross-Linking Degree on Hydrogels Using Surfactant Detergent as Template." Adanced Materials Research **284-286**: 1827-1830.
Chen, K. H., W. Mueannoom, et al. (2012). "Investigation into the effect of varying l-leucine concentration on the product characteristics of spray-dried liposome powders." J Pharm Pharmacol **64**(10): 1412-1424.
Chougule, M., B. Padhi, et al. (2007). "Nano-liposomal dry powder inhaler of tacrolimus: preparation, characterization, and pulmonary pharmacokinetics." Int J Nanomedicine **2**(4): 675-688.
Chougule, M., B. Padhi, et al. (2008). "Development of spray dried liposomal dry powder inhaler of Dapsone." AAPS PharmSciTech **9**(1): 47-53.
Chougule, M. B., B. K. Padhi, et al. (2006). "Nano-liposomal dry powder inhaler of Amiloride Hydrochloride." J Nanosci Nanotechnol **6**(9-10): 3001-3009.
Chow, A. H., H. H. Tong, et al. (2007). "Particle engineering for pulmonary drug delivery." Pharm Res **24**(3): 411-437.
Ciofu, O., T. Tolker-Nielsen, et al. (2014). "Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients." Adv Drug Deliv Rev.
Clayton, H., N. London, et al. (1991). "The effect of capsule composition on the biocompatibility of alginate-poly-1-lysine capsules." Journal of microencapsulation **8**(2): 221-233.
Codrons, V., F. Vanderbist, et al. (2004). "Impact of formulation and methods of pulmonary delivery on absorption of parathyroid hormone (1-34) from rat lungs." J Pharm Sci **93**(5): 1241-1252.
Cook, R. O., R. K. Pannu, et al. (2005). "Novel sustained release microspheres for pulmonary drug delivery." J Control Release **104**(1): 79-90.
Costerton, J. W., P. S. Stewart, et al. (1999). "Bacterial biofilms: a common cause of persistent infections." Science **284**(5418): 1318-1322.
Craig Dunbara, Gerhard Scheuchb, et al. (2002). "In vitro and in vivo dose delivery characteristics of large porous particles for inhalation." International Journal of Pharmaceutics **245**(1-2): 179-189.
Crapo, J. D., B. E. Barry, et al. (1982). "Cell number and cell characteristics of the normal human lung." Am Rev Respir Dis **126**(2): 332-337.
Creutzenberg, O., B. Bellmann, et al. (2012). "Change in agglomeration status and toxicokinetic fate of various nanoparticles in vivo following lung exposure in rats." Inhal Toxicol **24**(12): 821-830.
Crowder, T. M., J. A. Rosati, et al. (2002). "Fundamental effects of particle morphology on lung delivery: predictions of Stokes' law and the particular relevance to dry powder inhaler formulation and development." Pharm Res **19**(3): 239-245.
d'Angelo, I., C. Conte, et al. (2014). "Improving the efficacy of inhaled drugs in cystic fibrosis: Challenges and emerging drug delivery strategies." Adv Drug Deliv Rev **75C**: 92-111.
Daddario, M. K., J. K. Hagerman, et al. (2010). "Clinical perspective on aztreonam lysine for inhalation in patients with cystic fibrosis." Infect Drug Resist **3**: 123-132.
Dagar, S. (2007). Gibaldi's Drug Delivery Systems in Pharmaceutical care. Bethesda, MD, American Society of Health-System Pharmaceutics.
Dellamary, L. A., T. E. Tarara, et al. (2000). "Hollow porous particles in metered dose inhalers." Pharmaceutical research **17**(2): 168-174.

Denton, M., K. Kerr, et al. (2002). "Transmission of colistin-resistant *Pseudomonas aeruginosa* between patients attending a pediatric cystic fibrosis center." Pediatr Pulmonol **34**(4): 257-261.
Dershwitz, M., J. L. Walsh, et al. (2000). "Pharmacokinetics and pharmacodynamics of inhaled versus intravenous morphine in healthy volunteers." Anesthesiology **93**(3): 619-628.
Dhondikubeer, R., S. Bera, et al. (2012). "Antibacterial activity of amphiphilic tobramycin." The Journal of antibiotics **65**(10): 495-498.
Du, J., P. Du, et al. (2013). "Hydrogels for controlled pulmonary delivery." Therapeutic delivery **4**(10): 1293-1305.
Duceppe, N. and M. Tabrizian (2010). "Advances in using chitosan-based nanoparticles for in vitro and in vivo drug and gene delivery." Expert Opin Drug Deliv **7**(10): 1191-1207.
Edsbacker, S., P. Wollmer, et al. (2008). "Do airway clearance mechanisms influence the local and systemic effects of inhaled corticosteroids?" Pulm Pharmacol Ther **21**(2): 247-258.
Edwards, D. A., A. Ben-Jebria, et al. (1998). "Recent advances in pulmonary drug delivery using large, porous inhaled particles." J Appl Physiol **85**(2): 379-385.
Edwards, D. A., J. Hanes, et al. (1997). "Large porous particles for pulmonary drug delivery." Science **276**(5320): 1868-1871.
Edwards, D. A., J. Hanes, et al. (1997). "Large porous particles for pulmonary drug delivery." Science **276**(5320): 1868-1872.
Ehrhardt, C., J. Fiegel, et al. (2002). "Drug absorption by the respiratory mucosa: cell culture models and particulate drug carriers." J Aerosol Med **15**(2): 131-139.
Einarsson, O., G. P. Geba, et al. (1995). "Interleukin-11 in respiratory inflammation." Ann N Y Acad Sci **762**: 89-100; discussion 100-101.
El-Menshawe, S. F. and A. K. Hussein (2011). "Formulation and evaluation of meloxicam niosomes as vesicular carriers for enhanced skin delivery." Pharm Dev Technol.
El-Sherbiny, I. and H. Smyth (2011). "Smart Magnetically Responsive Hydrogel Nanoparticles Prepared by a Novel Aerosol-Assisted Method for Biomedical and Drug Delivery Applications." Journal of Nanomaterials **2011**.
El-Sherbiny, I. M., S. McGill, et al. (2010). "Swellable microparticles as carriers for sustained pulmonary drug delivery." J Pharm Sci **99**(5): 2343-2356.
El-Sherbiny, I. M. and H. D. Smyth (2010). "Biodegradable nano-micro carrier systems for sustained pulmonary drug delivery: (I) self-assembled nanoparticles encapsulated in respirable/swellable semi-IPN microspheres." Int J Pharm **395**(1-2): 132-141.
El-Sherbiny, I. M. and H. D. Smyth (2010). "Biodegradable nano-micro carrier systems for sustained pulmonary drug delivery:(I) self-assembled nanoparticles encapsulated in respirable/swellable semi-IPN microspheres." International journal of pharmaceutics **395**(1): 132-141.
El-Sherbiny, I. M. and H. D. Smyth (2010). "Poly (ethylene glycol)–carboxymethyl chitosan-based pH-responsive hydrogels: photo-induced synthesis, characterization, swelling, and in vitro evaluation as potential drug carriers." Carbohydrate research **345**(14): 2004-2012.
El-Sherbiny, I. M. and H. D. Smyth (2010). "Poly(ethylene glycol)-carboxymethyl chitosan-based pH-responsive hydrogels: photo-induced synthesis, characterization, swelling, and in vitro evaluation as potential drug carriers." Carbohydr Res **345**(14): 2004-2012.
El-Sherbiny, I. M. and H. D. Smyth (2011). "Controlled release pulmonary administration of curcumin using swellable biocompatible microparticles." Molecular pharmaceutics **9**(2): 269-280.
El-Sherbiny, I. M. and H. D. Smyth (2011). "Smart Magnetically Responsive Hydrogel Nanoparticles Prepared by a Novel Aerosol-Assisted Method for Biomedical and Drug Delivery Applications." J Nanomater **2011**(2011): 1-13.
El-Sherbiny, I. M. and H. D. Smyth (2012). "Controlled release pulmonary administration of curcumin using swellable biocompatible microparticles." Mol Pharm **9**(2): 269-280.

El-Sherbiny, I. M., S. McGill, et al. (2010). "Swellable microparticles as carriers for sustained pulmonary drug delivery." Journal of pharmaceutical sciences **99**(5): 2343-2356.
Fan, H. and A. K. Dash (2001). "Effect of cross-linking on the in vitro release kinetics of doxorubicin from gelatin implants." Int J Pharm **213**(1-2): 103-116.
Fanta, C. H. (2009). "Asthma." N Engl J Med **360**(10): 1002-1014.
Fernandes, C. A. and R. Vanbever (2009). "Preclinical models for pulmonary drug delivery." Expert Opin Drug Deliv **6**(11): 1231-1245.
fiegel, J., T. Brenza, et al. (2011). Controlled Treansport for pulmonary Drug Delivery. New York, Springer
Fiegel, J., C. Ehrhardt, et al. (2003). "Large porous particle impingement on lung epithelial cell monolayers--toward improved particle characterization in the lung." Pharm Res **20**(5): 788-796.
Fiegel, J., J. Fu, et al. (2004). "Poly(ether-anhydride) dry powder aerosols for sustained drug delivery in the lungs." J Control Release **96**(3): 411-423.
Flemming, H. C. and J. Wingender (2010). "The biofilm matrix." Nat Rev Microbiol **8**(9): 623-633.
Florindo, H. F., S. Pandit, et al. (2009). "New approach on the development of a mucosal vaccine against strangles: Systemic and mucosal immune responses in a mouse model." Vaccine **27**(8): 1230-1241.
Frederiksen, B., C. Koch, et al. (1999). "Changing epidemiology of *Pseudomonas aeruginosa* infection in Danish cystic fibrosis patients (1974-1995)." Pediatr Pulmonol **28**(3): 159-166.
Fu, J., J. Fiegel, et al. (2002). "New polymeric carriers for controlled drug delivery following inhalation or injection." Biomaterials **23**(22): 4425-4433.
Fux, C. A., J. W. Costerton, et al. (2005). "Survival strategies of infectious biofilms." Trends Microbiol **13**(1): 34-40.
Ganji, F. and E. Vasheghani-Farahani (2008). "Hydrogels in Controlled Drug Delivery Systems." Iranian Polymer Journal **18**(1): 63-88.
Garcia-Contreras, L. (2011). In Vivo Animal Models for Controlled-Relase Pulmonary Drug Delivery. New York, Springer
Gåserød, O., O. Smidsrød, et al. (1998). "Microcapsules of alginate-chitosan–I: a quantitative study of the interaction between alginate and chitosan." Biomaterials **19**(20): 1815-1825.
Gebert, A. and R. Pabst (1999). "M cells at locations outside the gut." Semin Immunol **11**(3): 165-170.
Gehr, P., M. Geiser, et al. (1993). "Surfactant and inhaled particles in the conducting airways: Structural, stereological, and biophysical aspects." Microscopy research technique **26**(5): 423-436.
Geiser, M. (2010). "Update on macrophage clearance of inhaled micro- and nanoparticles." J Aerosol Med Pulm Drug Deliv **23**(4): 207-217.
Geiser, M., B. Rothen-Rutishauser, et al. (2005). "Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells." Environ Health Perspect **113**(11): 1555-1560.
Geller, D. E. (2009). "Aerosol antibiotics in cystic fibrosis." Respiratory care **54**(5): 658-670.
Geller, D. E., M. W. Konstan, et al. (2007). "Novel tobramycin inhalation powder in cystic fibrosis subjects: pharmacokinetics and safety." Pediatr Pulmonol **42**(4): 307-313.
Gencay, M., M. Roth, et al. (2010). "Single and multiple viral infections in lower respiratory tract infection." Respiration **80**(6): 560-567.
George, A. M., P. M. Jones, et al. (2009). "Cystic fibrosis infections: treatment strategies and prospects." FEMS Microbiol Lett **300**(2): 153-164.
Giri, P. K. and G. K. Khuller (2008). "Is intranasal vaccination a feasible solution for tuberculosis?" Expert Rev Vaccines **7**(9): 1341-1356.

Giwercman, B., P. A. Lambert, et al. (1990). "Rapid emergence of resistance in *Pseudomonas aeruginosa* in cystic fibrosis patients due to *in-vivo* selection of stable partially derepressed beta-lactamase producing strains." J Antimicrob Chemother **26**(2): 247-259.
Giwercman, B., C. Meyer, et al. (1992). "High-level beta-lactamase activity in sputum samples from cystic fibrosis patients during antipseudomonal treatment." Antimicrob Agents Chemother **36**(1): 71-76.
Glover, W., H. K. Chan, et al. (2008). "Effect of particle size of dry powder mannitol on the lung deposition in healthy volunteers." Int J Pharm **349**(1-2): 314-322.
Glueck, R. (2001). "Review of intranasal influenza vaccine." Adv Drug Deliv Rev **51**(1-3): 203-211.
Gojgini, S., T. Tokatlian, et al. (2011). "Utilizing cell-matrix interactions to modulate gene transfer to stem cells inside hyaluronic acid hydrogels." Mol Pharm **8**(5): 1582-1591.
Gonzalez-Alvarez, M., I. Gonzalez-Alvarez, et al. (2013). "Hydrogels: an interesting strategy for smart drug delivery." Ther Deliv **4**(2): 157-160.
Gordon, S. B., R. Malamba, et al. (2008). "Inhaled delivery of 23-valent pneumococcal polysaccharide vaccine does not result in enhanced pulmonary mucosal immunoglobulin responses." Vaccine **26**(42): 5400-5406.
Gordon, S. B. and R. C. Read (2002). "Macrophage defences against respiratory tract infections." Br Med Bull **61**: 45-61.
Grenha, A., B. Seijo, et al. (2005). "Microencapsulated chitosan nanoparticles for lung protein delivery." Eur J Pharm Sci **25**(4-5): 427-437.
Gunbeyaz, M., A. Faraji, et al. (2010). "Chitosan based delivery systems for mucosal immunization against bovine herpesvirus 1 (BHV-1)." Eur J Pharm Sci **41**(3-4): 531-545.
Gupta, V. and F. Ahsan (2011). "Influence of PEI as a core modifying agent on PLGA microspheres of PGE(1), a pulmonary selective vasodilator." Int J Pharm **413**(1-2): 51-62.
Gupta, V., M. Davis, et al. (2011). "PLGA microparticles encapsulating prostaglandin E1-hydroxypropyl-beta-cyclodextrin (PGE1-HPbetaCD) complex for the treatment of pulmonary arterial hypertension (PAH)." Pharm Res **28**(7): 1733-1749.
Guvendiren, M., J. Burdick, et al. (2010). "Kinetic study of swelling-induced surface pattern formation and ordering in hydrogel films with depth-wise crosslinking gradient." Soft Matter **6**(9): 2044-2049.
Hancock, R. E., S. W. Farmer, et al. (1991). "Interaction of aminoglycosides with the outer membranes and purified lipopolysaccharide and OmpF porin of *Escherichia coli*." Antimicrob Agents Chemother **35**(7): 1309-1314.
Hassan, M. S. and R. W. Lau (2009). "Effect of particle shape on dry particle inhalation: study of flowability, aerosolization, and deposition properties." AAPS PharmSciTech **10**(4): 1252-1262.
Hathaway, L. J. and J. P. Kraehenbuhl (2000). "The role of M cells in mucosal immunity." Cell Mol Life Sci **57**(2): 323-332.
Haug, A. and O. Smidsrod (1965). "The effect of divalent metals on the properties of alginate solutions." Acta Chem. Scand **19**(2).
He, C., Y. Hu, et al. (2010). "Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles." Biomaterials **31**(13): 3657-3666.
Helgeson, M. E., S. C. Chapin, et al. (2011). "Hydrogel microparticles from lithographic processes: novel materials for fundamental and applied colloid science." Curr Opin Colloid Interface Sci **16**(2): 106-117.
Henke, M. O. and F. Ratjen (2007). "Mucolytics in cystic fibrosis." Paediatr Respir Rev **8**(1): 24-29.
Hennink, W. E. and C. F. van Nostrum (2002). "Novel crosslinking methods to design hydrogels." Adv Drug Deliv Rev **54**(1): 13-36.

Heyder, J. (2004). "Deposition of inhaled particles in the human respiratory tract and consequences for regional targeting in respiratory drug delivery." Proc Am Thorac Soc **1**(4): 315-320.
Hezaveh, H. and Muhamad, II (2012). "Impact of metal oxide nanoparticles on oral release properties of pH-sensitive hydrogel nanocomposites." Int J Biol Macromol **50**(5): 1334-1340.
Hezaveh, H., Muhamad, II, et al. (2012). "Swelling behaviour and controlled drug release from cross-linked kappa-carrageenan/NaCMC hydrogel by diffusion mechanism." J Microencapsul **29**(4): 368-379.
Hoare, T. and D. Kohane (2008). "Hydrogels in drug delivery: Progress and challenges." Polymer **49**: 1993-2007.
Hoffmann, N., T. B. Rasmussen, et al. (2005). "Novel mouse model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis." Infect Immun **73**(4): 2504-2514.
Hofmann, W. (2011). "Modelling inhaled particle deposition in the human lung—A review." Journal of Aerosol Science **42**(10): 693-724.
Hoiby, N. (2011). "Recent advances in the treatment of Pseudomonas aeruginosa infections in cystic fibrosis." BMC Med **9**: 32.
Hoiby, N., T. Bjarnsholt, et al. (2010). "Antibiotic resistance of bacterial biofilms." Int J Antimicrob Agents **35**(4): 322-332.
Hoiby, N., O. Ciofu, et al. (2010). "*Pseudomonas aeruginosa* biofilms in cystic fibrosis." Future Microbiol **5**(11): 1663-1674.
Hoiby, N., H. Krogh Johansen, et al. (2001). "*Pseudomonas aeruginosa* and the *in vitro* and *in vivo* biofilm mode of growth." Microbes Infect **3**(1): 23-35.
Houtmeyers, E., R. Gosselink, et al. (1999). "Regulation of mucociliary clearance in health and disease." Eur Respir J **13**(5): 1177-1188.
Houtmeyers, E., R. Gosselink, et al. (1999). "Regulation of mucociliary clearance in health and disease." European Respiratory Journal **13**(5): 1177-1188.
Howard, K. A., U. L. Rahbek, et al. (2006). "RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system." Mol Ther **14**(4): 476-484.
Huang, W. H., Z. J. Yang, et al. (2010). "Development of liposomal salbutamol sulfate dry powder inhaler formulation." Biol Pharm Bull **33**(3): 512-517.
Huynh, C. T., M. K. Nguyen, et al. (2011). "Synthesis, Characteristics and Potential Application of Poly(beta-Amino Ester Urethane)-Based Multiblock Co-Polymers as an Injectable, Biodegradable and pH/Temperature-Sensitive Hydrogel System." J Biomater Sci Polym Ed.
Hwang, S. M., D. D. Kim, et al. (2008). "Delivery of ofloxacin to the lung and alveolar macrophages via hyaluronan microspheres for the treatment of tuberculosis." J Control Release **129**(2): 100-106.
Ibrahim, B. M., M. D. Tsifansky, et al. (2011). "Challenges and advances in the development of inhalable drug formulations for cystic fibrosis lung disease." Expert opinion on drug delivery **8**(4): 451-466.
Ibrahim M.EI-Sherbiny, D. G. V., Dea Herrera, Hugh D.C. Smyth (2011). Controlled Pulmonary Drug Delivery. New York, Spring Science+Business Media.
Ikehata, M., R. Yumoto, et al. (2008). "Comparison of albumin uptake in rat alveolar type II and type I-like epithelial cells in primary culture." Pharm Res **25**(4): 913-922.
J Ducreux, R. V. (2007). "Crucial biopharmaceutical issues facing macromolecular candidates for inhalation: the role of macrophages in pulmonary protein clearance " Respiratory Drug Delivery Europe **1**: 31-41.
Jalal, S., O. Ciofu, et al. (2000). "Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients." Antimicrob Agents Chemother **44**(3): 710-712.

Jana, S. and J. K. Deb (2006). "Molecular understanding of aminoglycoside action and resistance." Appl Microbiol Biotechnol **70**(2): 140-150.
Jelsbak, L., H. K. Johansen, et al. (2007). "Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis patients." Infect Immun **75**(5): 2214-2224.
Jensen, D. K., L. B. Jensen, et al. (2012). "Design of an inhalable dry powder formulation of DOTAP-modified PLGA nanoparticles loaded with siRNA." J Control Release **157**(1): 141-148.
Johansen, H. K., S. M. Moskowitz, et al. (2008). "Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients." J Cyst Fibros **7**(5): 391-397.
John, T. A., S. M. Vogel, et al. (2001). "Evidence for the role of alveolar epithelial gp60 in active transalveolar albumin transport in the rat lung." J Physiol **533**(Pt 2): 547-559.
Joshi, M. R. and A. Misra (2001). "Liposomal budesonide for dry powder inhaler: preparation and stabilization." AAPS PharmSciTech **2**(4): 25.
Joshi, M. R. and A. N. Misra (1999). "Liposomes of terbutaline sulphate: in vitro and in vivo studies." Indian J Exp Biol **37**(9): 881-887.
Julie Todoroff, R. V. (2011). "Fate of nanomedicines in the lungs." Current Opinion in Colloid & Interface Science **16**(3): 246-254.
Juntapram, K., N. Praphairaksit, et al. (2012). "Electrosprayed polyelectrolyte complexes between mucoadhesive N,N,N,-trimethylchitosan-homocysteine thiolactone and alginate/carrageenan for camptothecin delivery." Carbohydr Polym **90**(4): 1469-1479.
Karathanasis, E., R. Bhavane, et al. (2007). "Glucose-sensing pulmonary delivery of human insulin to the systemic circulation of rats." Int J Nanomedicine **2**(3): 501-513.
Kim, B., S. H. Lim, et al. (2009). "Preparation and characterization of pH-sensitive anionic hydrogel microparticles for oral protein-delivery applications." J Biomater Sci Polym Ed **20**(4): 427-436.
Kim, D.-H. and D. C. Martin (2006). "Sustained release of dexamethasone from hydrophilic matrices using PLGA nanoparticles for neural drug delivery." Biomaterials **27**(15): 3031-3037.
Kim, D. Y., A. Sato, et al. (2011). "The airway antigen sampling system: respiratory M cells as an alternative gateway for inhaled antigens." J Immunol **186**(7): 4253-4262.
Kim, H., J. Lee, et al. (2011). "Albumin-coated porous hollow poly(lactic-co-glycolic acid) microparticles bound with palmityl-acylated exendin-4 as a long-acting inhalation delivery system for the treatment of diabetes." Pharm Res **28**(8): 2008-2019.
Kim, I., H. J. Byeon, et al. (2012). "Doxorubicin-loaded highly porous large PLGA microparticles as a sustained- release inhalation system for the treatment of metastatic lung cancer." Biomaterials **33**(22): 5574-5583.
Kim, S., A. E. English, et al. (2009). "Surface elasticity and charge concentration-dependent endothelial cell attachment to copolymer polyelectrolyte hydrogel." Acta Biomater **5**(1): 144-151.
Kirkby, S., K. Novak, et al. (2011). "Aztreonam (for inhalation solution) for the treatment of chronic lung infections in patients with cystic fibrosis: an evidence-based review." Core Evid **6**: 59-66.
Klepser, M. E., E. J. Ernst, et al. (1998). "Evaluation of endpoints for antifungal susceptibility determinations with LY303366." Antimicrob Agents Chemother **42**(6): 1387-1391.
Ko, I. K., A. Ziady, et al. (2008). "Acid-degradable cationic methacrylamide polymerized in the presence of plasmid DNA as tunable non-viral gene carrier." Biomaterials **29**(28): 3872-3881.

Kong, X., W. Zhang, et al. (2007). "Respiratory syncytial virus infection in Fischer 344 rats is attenuated by short interfering RNA against the RSV-NS1 gene." Genet Vaccines Ther **5**: 4.

Kotra, L. P., J. Haddad, et al. (2000). "Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance." Antimicrob Agents Chemother **44**(12): 3249-3256.

Kraehenbuhl, J. P. and M. R. Neutra (2000). "Epithelial M cells: differentiation and function." Annu Rev Cell Dev Biol **16**: 301-332.

Kulkarni, R. V., R. Boppana, et al. (2012). "pH-responsive interpenetrating network hydrogel beads of poly(acrylamide)-g-carrageenan and sodium alginate for intestinal targeted drug delivery: synthesis, in vitro and in vivo evaluation." J Colloid Interface Sci **367**(1): 509-517.

Kushwaha, S. K., P. Saxena, et al. (2012). "Stimuli sensitive hydrogels for ophthalmic drug delivery: A review." Int J Pharm Investig **2**(2): 54-60.

Kwon, M. J., J. H. Bae, et al. (2007). "Long acting porous microparticle for pulmonary protein delivery." International journal of pharmaceutics **333**(1): 5-9.

L, C.-C. (2011). In vivo animal models for controlled-release pulmonary drug delivery. NY US, Springer.

Lasic, D. (1995). "Applications of liposomes." Handbook of biological physics **1**: 491-519.

Lee, B., J. A. Haagensen, et al. (2005). "Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis." J Clin Microbiol **43**(10): 5247-5255.

Lee, J. and K. Y. Lee (2009). "Local and sustained vascular endothelial growth factor delivery for angiogenesis using an injectable system." Pharmaceutical research **26**(7): 1739-1744.

Lee, K. Y. and D. J. Mooney (2012). "Alginate: properties and biomedical applications." Progress in polymer science **37**(1): 106-126.

Lemke, C. D., J. B. Graham, et al. (2011). "Chitosan is a surprising negative modulator of cytotoxic CD8+ T cell responses elicited by adenovirus cancer vaccines." Mol Pharm **8**(5): 1652-1661.

Lin, C.-E., Y. Deng Jr, et al. (2004). "Electrophoretic behavior and p< i> K</i>< sub> a</sub> determination of quinolones with a piperazinyl substituent by capillary zone electrophoresis." Journal of Chromatography A **1051**(1): 283-290.

Linsuwanon, P., S. Payungporn, et al. (2009). "High prevalence of human rhinovirus C infection in Thai children with acute lower respiratory tract disease." J Infect **59**(2): 115-121.

Liu, C., J. Shi, et al. (2013). "In-vitro and in-vivo evaluation of ciprofloxacin liposome for pulmonary administration." Drug development and industrial pharmacy(0): 1-7.

Liu, Y., A. Ibricevic, et al. (2009). "Impact of hydrogel nanoparticle size and functionalization on in vivo behavior for lung imaging and therapeutics." Mol Pharm **6**(6): 1891-1902.

Liu, Z., J. T. Robinson, et al. (2008). "PEGylated nanographene oxide for delivery of water-insoluble cancer drugs." Journal of the American Chemical Society **130**(33): 10876-10877.

Lo, Y. L., C. Y. Hsu, et al. (2013). "pH-and thermo-sensitive pluronic/poly(acrylic acid) in situ hydrogels for sustained release of an anticancer drug." J Drug Target **21**(1): 54-66.

Lombry, C., C. Bosquillon, et al. (2002). "Confocal imaging of rat lungs following intratracheal delivery of dry powders or solutions of fluorescent probes." J Control Release **83**(3): 331-341.

Lombry, C., D. A. Edwards, et al. (2004). "Alveolar macrophages are a primary barrier to pulmonary absorption of macromolecules." Am J Physiol Lung Cell Mol Physiol **286**(5): L1002-1008.

Londahl, J., J. Pagels, et al. (2008). "Deposition of biomass combustion aerosol particles in the human respiratory tract." Inhal Toxicol **20**(10): 923-933.

Louey MD , G.-C. L. (2004). "Controlled release products for respiratory delivery." Am Pharm Rev **7**: 82-87.
Lu, D. and A. J. Hickey (2007). "Pulmonary vaccine delivery." Expert Rev Vaccines **6**(2): 213-226.
Luppi, B., F. Bigucci, et al. (2010). "Chitosan-based hydrogels for nasal drug delivery: from inserts to nanoparticles." Expert Opin Drug Deliv **7**(7): 811-828.
Luten, J., M. J. van Steenbergen, et al. (2008). "Degradable PEG-folate coated poly(DMAEA-*co*-BA)phosphazene-based polyplexes exhibit receptor-specific gene expression." Eur J Pharm Sci **33**(3): 241-251.
MacLeod, D. L., L. E. Nelson, et al. (2000). "Aminoglycoside-resistance mechanisms for cystic fibrosis *Pseudomonas aeruginosa* isolates are unchanged by long-term, intermittent, inhaled tobramycin treatment." J Infect Dis **181**(3): 1180-1184.
Magnet, S. and J. S. Blanchard (2005). "Molecular insights into aminoglycoside action and resistance." Chem Rev **105**(2): 477-498.
Mah, T. F. and G. A. O'Toole (2001). "Mechanisms of biofilm resistance to antimicrobial agents." Trends Microbiol **9**(1): 34-39.
Mahavir Chougule, Bijay Padhi, et al. (2007). "Nano-liposomal dry powder inhaler of tacrolimus: Preparation, characterization, and pulmonary pharmacokinetics." International Journal of Nanomedicine **2**(4): 675-688.
Mahmoudi, Z. N., S. B. Upadhye, et al. (2014). "In Vitro Characterization of a Novel Polymeric System for Preparation of Amorphous Solid Drug Dispersions." The AAPS journal: 1-13.
Mahmud, A. and D. E. Discher (2011). "Lung vascular targeting through inhalation delivery: insight from filamentous viruses and other shapes." IUBMB Life **63**(8): 607-612.
Makino, K., N. Yamamoto, et al. (2003). "Phagocytic uptake of polystyrene microspheres by alveolar macrophages: effects of the size and surface properties of the microspheres " Colloids and Surfaces B: Biointerfaces **27**: 33-39.
Mansour, H. M., Y.-S. Rhee, et al. (2009). "Nanomedicine in pulmonary delivery." International journal of nanomedicine **4**: 299.
Mansour, H. M., Y. S. Rhee, et al. (2009). "Nanomedicine in pulmonary delivery." Int J Nanomedicine **4**: 299-319.
Matsukawa, Y., V. H. Lee, et al. (1997). "Size-dependent dextran transport across rat alveolar epithelial cell monolayers." J Pharm Sci **86**(3): 305-309.
McCoy, K. S., A. L. Quittner, et al. (2008). "Inhaled aztreonam lysine for chronic airway Pseudomonas aeruginosa in cystic fibrosis." Am J Respir Crit Care Med **178**(9): 921-928.
McGill, S. L. and H. D. Smyth (2010). "Disruption of the mucus barrier by topically applied exogenous particles." Mol Pharm **7**(6): 2280-2288.
McKeage, K. (2013). "Tobramycin inhalation powder: a review of its use in the treatment of chronic *Pseudomonas aeruginosa* infection in patients with cystic fibrosis." Drugs **73**(16): 1815-1827.
Meenach, S. A., Y. J. Kim, et al. (2012). "Synthesis, optimization, and characterization of camptothecin-loaded acetalated dextran porous microparticles for pulmonary delivery." Mol Pharm **9**(2): 290-298.
Menzel, M., B. Muellinger, et al. (2005). "Inhalative vaccination with pneumococcal polysaccharide in healthy volunteers." Vaccine **23**(43): 5113-5119.
Mercer, R. R., M. L. Russell, et al. (1994). "Cell number and distribution in human and rat airways." Am J Respir Cell Mol Biol **10**(6): 613-624.
Mert, O., S. K. Lai, et al. (2012). "A poly(ethylene glycol)-based surfactant for formulation of drug-loaded mucus penetrating particles." J Control Release **157**(3): 455-460.
Mesaros, N., P. Nordmann, et al. (2007). "*Pseudomonas aeruginosa:* resistance and therapeutic options at the turn of the new millennium." Clin Microbiol Infect **13**(6): 560-578.

Mihaila, S. M., A. K. Gaharwar, et al. (2012). "Photocrosslinkable Kappa-Carrageenan Hydrogels for Tissue Engineering Applications." Adv Healthc Mater.
Mingeot-Leclercq, M. P., Y. Glupczynski, et al. (1999). "Aminoglycosides: activity and resistance." Antimicrob Agents Chemother **43**(4): 727-737.
Minne, A., J. Louahed, et al. (2007). "The delivery site of a monovalent influenza vaccine within the respiratory tract impacts on the immune response." Immunology **122**(3): 316-325.
Mobus, K., J. Siepmann, et al. (2012). "Zinc-alginate microparticles for controlled pulmonary delivery of proteins prepared by spray-drying." Eur J Pharm Biopharm **81**(1): 121-130.
Monforte, V., A. Lopez-Sanchez, et al. (2013). "Prophylaxis with nebulized liposomal amphotericin B for Aspergillus infection in lung transplant patients does not cause changes in the lipid content of pulmonary surfactant." J Heart Lung Transplant **32**(3): 313-319.
Morfeld, P., S. Treumann, et al. (2012). "Deposition behavior of inhaled nanostructured TiO2 in rats: fractions of particle diameter below 100 nm (nanoscale) and the slicing bias of transmission electron microscopy." Inhal Toxicol **24**(14): 939-951.
Morimoto, K., K. Metsugi, et al. (2001). "Effects of low-viscosity sodium hyaluronate preparation on the pulmonary absorption of rh-insulin in rats." Drug Dev Ind Pharm **27**(4): 365-371.
Moss, R. B. (2009). "Infection, inflammation, and the downward spiral of cystic fibrosis lung disease." The Journal of pediatrics **154**(2): 162-163.
Moss, R. B., C. Milla, et al. (2007). "Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial." Hum Gene Ther **18**(8): 726-732.
Murata, M., T. Yonamine, et al. (2013). "Surface modification of liposomes using polymer-wheat germ agglutinin conjugates to improve the absorption of peptide drugs by pulmonary administration." J Pharm Sci **102**(4): 1281-1289.
Musante, C. J., J. D. Schroeter, et al. (2002). "Factors affecting the deposition of inhaled porous drug particles." J Pharm Sci **91**(7): 1590-1600.
Muttil, P., C. Prego, et al. (2010). "Immunization of guinea pigs with novel hepatitis B antigen as nanoparticle aggregate powders administered by the pulmonary route." AAPS J **12**(3): 330-337.
Nagpal, K., S. K. Singh, et al. (2010). "Chitosan nanoparticles: a promising system in novel drug delivery." Chem Pharm Bull (Tokyo) **58**(11): 1423-1430.
Namdeo, M., S. K. Bajpai, et al. (2009). "Preparation of a magnetic-field-sensitive hydrogel and preliminary study of its drug release behavior." J Biomater Sci Polym Ed **20**(12): 1747-1761.
Nave, R., R. Fisher, et al. (2006). "In Vitro metabolism of ciclesonide in human lung and liver precision-cut tissue slices." Biopharm Drug Dispos **27**(4): 197-207.
Nguyen, J., T. W. Steele, et al. (2008). "Fast degrading polyesters as siRNA nano-carriers for pulmonary gene therapy." J Control Release **132**(3): 243-251.
Nguyen, X. C., J. D. Herberger, et al. (2004). "Protein powders for encapsulation: a comparison of spray-freeze drying and spray drying of darbepoetin alfa." Pharm Res **21**(3): 507-514.
Niamlang, S. and A. Sirivat (2009). "Electric field assisted transdermal drug delivery from salicylic acid-loaded polyacrylamide hydrogels." Drug Deliv **16**(7): 378-388.
Nickel, J. C., I. Ruseska, et al. (1985). "Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material." Antimicrob Agents Chemother **27**(4): 619-624.
Nielsen, E. J., J. M. Nielsen, et al. (2010). "Pulmonary gene silencing in transgenic EGFP mice using aerosolised chitosan/siRNA nanoparticles." Pharm Res **27**(12): 2520-2527.
NIH. (2012). "How Is COPD Treated?".

Niranjan, R., C. Koushik, et al. (2013). "A novel injectable temperature-sensitive zinc doped chitosan/beta-glycerophosphate hydrogel for bone tissue engineering." Int J Biol Macromol **54**: 24-29.
Niven, R. W. (1995). "Delivery of biotherapeutics by inhalation aerosol." Crit Rev Ther Drug Carrier Syst **12**(2-3): 151-231.
O'Hara, P. and A. J. Hickey (2000). "Respirable PLGA microspheres containing rifampicin for the treatment of tuberculosis: manufacture and characterization." Pharmaceutical research **17**(8): 955-961.
Oberdorster, G. (2001). "Pulmonary effects of inhaled ultrafine particles." Int Arch Occup Environ Health **74**(1): 1-8.
Oh, Y. J., J. Lee, et al. (2011). "Preparation of budesonide-loaded porous PLGA microparticles and their therapeutic efficacy in a murine asthma model." J Control Release **150**(1): 56-62.
Ong, H. X., D. Traini, et al. (2011). "Epithelial profiling of antibiotic controlled release respiratory formulations." Pharm Res **28**(9): 2327-2338.
Orive, G., S. Ponce, et al. (2002). "Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginates." Biomaterials **23**(18): 3825-3831.
Otterlei, M., K. Østgaard, et al. (1991). "Induction of cytokine production from human monocytes stimulated with alginate." Journal of Immunotherapy **10**(4): 286-291.
Panos, I., N. Acosta, et al. (2008). "New drug delivery systems based on chitosan." Curr Drug Discov Technol **5**(4): 333-341.
Park, J. H., G. Saravanakumar, et al. (2010). "Targeted delivery of low molecular drugs using chitosan and its derivatives." Adv Drug Deliv Rev **62**(1): 28-41.
Park, Y. D., N. Tirelli, et al. (2003). "Photopolymerized hyaluronic acid-based hydrogels and interpenetrating networks." Biomaterials **24**(6): 893-900.
Parra, S. C., R. Burnette, et al. (1986). "Zonal distribution of alveolar macrophages, type II pneumonocytes, and alveolar septal connective tissue gaps in adult human lungs." Am Rev Respir Dis **133**(5): 908-912.
Patton, J. S. (1996). "Mechanisms of macromolecule absorption by the lungs." Advanced Drug Delivery Reviews **19**(1): 3-36.
Patton, J. S. and P. R. Byron (2007). "Inhaling medicines: delivering drugs to the body through the lungs." Nat Rev Drug Discov **6**(1): 67-74.
Patton, J. S. and P. R. Byron (2007). "Inhaling medicines: delivering drugs to the body through the lungs." Nature Reviews Drug Discovery **6**(1): 67-74.
Patton, J. S., C. S. Fishburn, et al. (2004). "The lungs as a portal of entry for systemic drug delivery." Proc Am Thorac Soc **1**(4): 338-344.
Pawar, D., A. K. Goyal, et al. (2010). "Evaluation of mucoadhesive PLGA microparticles for nasal immunization." AAPS J **12**(2): 130-137.
Pearlman, D. S., P. Chervinsky, et al. (1992). "A comparison of salmeterol with albuterol in the treatment of mild-to-moderate asthma." N Engl J Med **327**(20): 1420-1425.
Peiris, J. S., Y. Guan, et al. (2004). "Severe acute respiratory syndrome." Nat Med **10**(12 Suppl): S88-97.
Perez, R. A., J. E. Won, et al. (2012). "Naturally and synthetic smart composite biomaterials for tissue regeneration." Adv Drug Deliv Rev.
Pier, G. B. (2012). "The challenges and promises of new therapies for cystic fibrosis." J Exp Med **209**(7): 1235-1239.
Pilcer, G., T. Sebti, et al. (2006). "Formulation and characterization of lipid-coated tobramycin particles for dry powder inhalation." Pharmaceutical research **23**(5): 931-940.
Podczeck, F. and B. Jones (2004). Pharmaceutical Capsules, 2nd edn., Pharmaceutical Press.
Poonam Sheth, P. B. M. (2011). Controlled Pulmonary Drug Delivery. New York, Springer Science+Business Media.

Poonam Sheth, P. B. M. (2011, 244). Controlled Pulmonary Drug Delivery. New York, Springer Science+Business Media.
Popa, E. G., S. G. Caridade, et al. (2013). "Chondrogenic potential of injectable kappa-carrageenan hydrogel with encapsulated adipose stem cells for cartilage tissue-engineering applications." J Tissue Eng Regen Med.
Popa, E. G., M. E. Gomes, et al. (2011). "Cell delivery systems using alginate--carrageenan hydrogel beads and fibers for regenerative medicine applications." Biomacromolecules **12**(11): 3952-3961.
Popielarski, S. R., S. H. Pun, et al. (2005). "A nanoparticle-based model delivery system to guide the rational design of gene delivery to the liver. 1. Synthesis and characterization." Bioconjug Chem **16**(5): 1063-1070.
Potera, C. (2010). "Antibiotic Resistance: Biofilm Dispersing Agent Rejuvenates Older Antibiotics." Environmental Health Perspectives **118**(7): A288.
Ratjen, F. (2007). "New pulmonary therapies for cystic fibrosis." Curr Opin Pulm Med **13**(6): 541-546.
Ratjen, F. A. (2009). "Cystic fibrosis: pathogenesis and future treatment strategies." Respir Care **54**(5): 595-605.
Rawat, A., Q. H. Majumder, et al. (2008). "Inhalable large porous microspheres of low molecular weight heparin: in vitro and in vivo evaluation." J Control Release **128**(3): 224-232.
Reznikov, L. R., M. H. Abou Alaiwa, et al. (2014). "Antibacterial properties of the CFTR potentiator ivacaftor." J Cyst Fibros **13**(5): 515-519.
Roberts, J. A. and J. Lipman (2006). "Antibacterial dosing in intensive care." Clinical Pharmacokinetics **45**(8): 755-773.
Rossi, S. E., J. J. Erasmus, et al. (2000). "Pulmonary drug toxicity: radiologic and pathologic manifestations." Radiographics **20**(5): 1245-1259.
Rossi, S. E., J. J. Erasmus, et al. (2000). "Pulmonary Drug Toxicity: Radiologic and Pathologic Manifestations 1." Radiographics **20**(5): 1245-1259.
Rouse, J. J., T. L. Whateley, et al. (2007). "Controlled drug delivery to the lung: Influence of hyaluronic acid solution conformation on its adsorption to hydrophobic drug particles." Int J Pharm **330**(1-2): 175-182.
Roy, I. and N. Vij (2010). "Nanodelivery in airway diseases: challenges and therapeutic applications." Nanomedicine **6**(2): 237-244.
Ruff, L. E., E. A. Mahmoud, et al. (2013). "Antigen-loaded pH-sensitive hydrogel microparticles are taken up by dendritic cells with no requirement for targeting antibodies." Integr Biol (Camb) **5**(1): 195-203.
Ruge, C. A., J. Kirch, et al. (2013). "Pulmonary drug delivery: from generating aerosols to overcoming biological barriers—therapeutic possibilities and technological challenges." The Lancet Respiratory Medicine.
Rytting, E., J. Nguyen, et al. (2008). "Biodegradable polymeric nanocarriers for pulmonary drug delivery." Expert Opin Drug Deliv **5**(6): 629-639.
Rytting, E., J. Nguyen, et al. (2008). "Biodegradable polymeric nanocarriers for pulmonary drug delivery."
Sahana, D., G. Mittal, et al. (2008). "PLGA nanoparticles for oral delivery of hydrophobic drugs: influence of organic solvent on nanoparticle formation and release behavior in vitro and in vivo using estradiol as a model drug." Journal of pharmaceutical sciences **97**(4): 1530-1542.
Sahib, M. N., Y. Darwis, et al. (2011). "Rehydrated sterically stabilized phospholipid nanomicelles of budesonide for nebulization: physicochemical characterization and in vitro, in vivo evaluations." Int J Nanomedicine **6**: 2351-2366.

Saiman, L., F. Mehar, et al. (1996). "Antibiotic susceptibility of multiply resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis, including candidates for transplantation." Clin Infect Dis **23**(3): 532-537.
Sakagami, M., W. Kinoshita, et al. (2002). "Mucoadhesive beclomethasone microspheres for powder inhalation: their pharmacokinetics and pharmacodynamics evaluation." J Control Release **80**(1-3): 207-218.
Salgueiro, A. M., A. L. Daniel-da-Silva, et al. (2013). "kappa-Carrageenan hydrogel nanocomposites with release behavior mediated by morphological distinct Au nanofillers." Carbohydr Polym **91**(1): 100-109.
Samaranayake, L. P. (2006). Essential Microbiology for Dentistry. Edinburgh, Churchill Livingstone.
Sanjar, S. and J. Matthews (2001). "Treating systemic diseases via the lung." J Aerosol Med **14 Suppl 1**: S51-58.
Satarkar, N. S. and J. Z. Hilt (2008). "Magnetic hydrogel nanocomposites for remote controlled pulsatile drug release." J Control Release **130**(3): 246-251.
Scalia, S., R. Salama, et al. (2012). "Preparation and in vitro evaluation of salbutamol-loaded lipid microparticles for sustained release pulmonary therapy." Journal of microencapsulation **29**(3): 225-233.
Schneider, G. B., A. English, et al. (2004). "The effect of hydrogel charge density on cell attachment." Biomaterials **25**(15): 3023-3028.
Sechriest, V. F., Y. J. Miao, et al. (2000). "GAG-augmented polysaccharide hydrogel: A novel biocompatible and biodegradable material to support chondrogenesis." Journal of biomedical materials research **49**(4): 534-541.
Seguin, J., L. Brulle, et al. (2013). "Liposomal encapsulation of the natural flavonoid fisetin improves bioavailability and antitumor efficacy." Int J Pharm **444**(1-2): 146-154.
Segura, T., B. C. Anderson, et al. (2005). "Crosslinked hyaluronic acid hydrogels: a strategy to functionalize and pattern." Biomaterials **26**(4): 359-371.
Selvam, P., I. M. El-Sherbiny, et al. (2011). "Swellable hydrogel particles for controlled release pulmonary administration using propellant-driven metered dose inhalers." J Aerosol Med Pulm Drug Deliv **24**(1): 25-34.
Selvam, P., I. M. El-Sherbiny, et al. (2011). "Swellable hydrogel particles for controlled release pulmonary administration using propellant-driven metered dose inhalers." Journal of aerosol medicine and pulmonary drug delivery **24**(1): 25-34.
Semmler-Behnke, M., S. Takenaka, et al. (2007). "Efficient elimination of inhaled nanoparticles from the alveolar region: evidence for interstitial uptake and subsequent reentrainment onto airways epithelium." Environ Health Perspect **115**(5): 728-733.
Semmler, M., J. Seitz, et al. (2004). "Long-term clearance kinetics of inhaled ultrafine insoluble iridium particles from the rat lung, including transient translocation into secondary organs." Inhal Toxicol **16**(6-7): 453-459.
Serisier, D. (2012). "Inhaled antibiotics for lower respiratory tract infections: focus on ciprofloxacin." Drugs of today (Barcelona, Spain: 1998) **48**(5): 339-351.
Shah, S. P. and A. Misra (2004). "Development of liposomal amphotericin B dry powder inhaler formulation." Drug Deliv **11**(4): 247-253.
Shahiwala, A. and A. Misra (2005). "A preliminary pharmacokinetic study of liposomal leuprolide dry powder inhaler: a technical note." AAPS PharmSciTech **6**(3): E482-486.
Shak, S., D. J. Capon, et al. (1990). "Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum." Proc Natl Acad Sci U S A **87**(23): 9188-9192.
Sham, J. O., Y. Zhang, et al. (2004). "Formulation and characterization of spray-dried powders containing nanoparticles for aerosol delivery to the lung." Int J Pharm **269**(2): 457-467.
Sharma, G., W. Mueannoom, et al. (2013). "In vitro characterisation of terbutaline sulphate particles prepared by thermal ink-jet spray freeze drying." Int J Pharm.

Sharma, S., T. K. Mukkur, et al. (2009). "Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems." J Pharm Sci **98**(3): 812-843.
Shaul, P., K. D. Green, et al. (2011). "Assessment of 6'- and 6'"-N-acylation of aminoglycosides as a strategy to overcome bacterial resistance." Org Biomol Chem **9**(11): 4057-4063.
Shu, Z., X. Liu, et al. (2004). "In situ crosslinkable hyaluronan hydrogels for tissue engineering." Biomaterials **25**(7-8): 1339-1348.
Sikorski, P., F. Mo, et al. (2007). "Evidence for egg-box-compatible interactions in calcium-alginate gels from fiber X-ray diffraction." Biomacromolecules **8**(7): 2098-2103.
Singh, G. and G. Poochikian (2011). Development and Approval of Inhaled Respiratory Drugs: A US Regulatory Science Perspective. New York, Springer
Singh GLP and P. G. (2011). Development and approval of inhaled respiratory drugs: a US regulatory science perspective. NY, US, Springer.
Singh, S., F. Topuz, et al. (2013). "Embedding of Active Proteins and Living Cells in Redox-Sensitive Hydrogels and Nanogels through Enzymatic Cross-Linking." Angew Chem Int Ed Engl **52**(10): 3000-3003.
Sioud, M. (2005). "On the delivery of small interfering RNAs into mammalian cells." Expert Opin Drug Deliv **2**(4): 639-651.
Sivadas, N. and S. A. Cryan (2011). "Inhalable, bioresponsive microparticles for targeted drug delivery in the lungs." J Pharm Pharmacol **63**(3): 369-375.
Smyth, H. D. and A. J. Hickey (2005). "Carriers in drug powder delivery." American Journal of Drug Delivery **3**(2): 117-132.
Son, Y. J. and J. T. McConville (2008). "Advancements in dry powder delivery to the lung." Drug Dev Ind Pharm **34**(9): 948-959.
Soon-Shiong, P., M. Otterlie, et al. (1991). An immunologic basis for the fibrotic reaction to implanted microcapsules. Transplantation Proceedings.
Stass, H., B. Weimann, et al. (2013). "Tolerability and Pharmacokinetic Properties of Ciprofloxacin Dry Powder for Inhalation in Patients With Cystic Fibrosis: A Phase I, Randomized, Dose-Escalation Study." Clinical therapeutics **35**(10): 1571-1581.
Steimer, A., E. Haltner, et al. (2005). "Cell culture models of the respiratory tract relevant to pulmonary drug delivery." J Aerosol Med **18**(2): 137-182.
Stewart, M. B., S. R. Gray, et al. (2014). "The role of poly-M and poly-GM sequences in the metal-mediated assembly of alginate gels." Carbohydrate Polymers.
Stewart, P. S. (1996). "Theoretical aspects of antibiotic diffusion into microbial biofilms." Antimicrob Agents Chemother **40**(11): 2517-2522.
Stewart, P. S. and J. W. Costerton (2001). "Antibiotic resistance of bacteria in biofilms." Lancet **358**(9276): 135-138.
Suk, J. S., A. J. Kim, et al. (2014). "Lung gene therapy with highly compacted DNA nanoparticles that overcome the mucus barrier." J Control Release **178**: 8-17.
Sung, J. C., D. J. Padilla, et al. (2009). "Formulation and pharmacokinetics of self-assembled rifampicin nanoparticle systems for pulmonary delivery." Pharmaceutical research **26**(8): 1847-1855.
Sung, J. C., B. L. Pulliam, et al. (2007). "Nanoparticles for drug delivery to the lungs." Trends Biotechnol **25**(12): 563-570.
Surendrakumar, K., G. Martyn, et al. (2003). "Sustained release of insulin from sodium hyaluronate based dry powder formulations after pulmonary delivery to beagle dogs." Journal of Controlled Release **91**(3): 385-394.
Surendrakumar, K., G. P. Martyn, et al. (2003). "Sustained release of insulin from sodium hyaluronate based dry powder formulations after pulmonary delivery to beagle dogs." J Control Release **91**(3): 385-394.

Szaff, M., N. Hoiby, et al. (1983). "Frequent antibiotic therapy improves survival of cystic fibrosis patients with chronic *Pseudomonas aeruginosa* infection." Acta Paediatr Scand **72**(5): 651-657.
Takeuchi, H., H. Yamamoto, et al. (2001). "Mucoadhesive nanoparticulate systems for peptide drug delivery." Adv Drug Deliv Rev **47**(1): 39-54.
Theravance (2013). "Theravance Reports Fourth Quarter and Full Year 2012 Financial Results." Theravance, Inc.
Thomas, C., V. Gupta, et al. (2010). "Particle size influences the immune response produced by hepatitis B vaccine formulated in inhalable particles." Pharm Res **27**(5): 905-919.
Thomas, C., A. Rawat, et al. (2011). "Aerosolized PLA and PLGA nanoparticles enhance humoral, mucosal and cytokine responses to hepatitis B vaccine." Mol Pharm **8**(2): 405-415.
Thomas, D. A., M. A. Myers, et al. (1991). "Acute effects of liposome aerosol inhalation on pulmonary function in healthy human volunteers." Chest **99**(5): 1268-1270.
Tin, S., K. R. Sakharkar, et al. (2009). "Activity of Chitosans in combination with antibiotics in *Pseudomonas aeruginosa*." Int J Biol Sci **5**(2): 153-160.
Todoroff, J. and R. Vanbever (2011). "fate of nanomedicine in the lungs." Current Opinion in Colloid & Interface Science **16**(3): 246-254.
Tosi, M. F., A. van Heeckeren, et al. (2004). "Effect of Pseudomonas-induced chronic lung inflammation on specific cytotoxic T-cell responses to adenoviral vectors in mice." Gene Ther **11**(19): 1427-1433.
Tre-Hardy, M., H. Traore, et al. (2009). "Evaluation of long-term co-administration of tobramycin and clarithromycin in a mature biofilm model of cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*." Int J Antimicrob Agents **34**(4): 370-374.
Tsapis, N., D. Bennett, et al. (2002). "Trojan particles: large porous carriers of nanoparticles for drug delivery." Proceedings of the National Academy of Sciences **99**(19): 12001-12005.
Tseng, B. S., W. Zhang, et al. (2013). "The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin." Environ Microbiol **15**(10): 2865-2878.
Tsifansky, M. D., Y. Yeo, et al. (2008). "Microparticles for inhalational delivery of antipseudomonal antibiotics." AAPS J **10**(2): 254-260.
Tu, J., L. Wang, et al. (2001). "Formulation and pharmacokinetic studies of acyclovir controlled-release capsules." Drug Dev Ind Pharm **27**(7): 687-692.
Tunek, A., K. Sjodin, et al. (1997). "Reversible formation of fatty acid esters of budesonide, an antiasthma glucocorticoid, in human lung and liver microsomes." Drug Metab Dispos **25**(11): 1311-1317.
Ungaro, F., G. De Rosa, et al. (2006). "Cyclodextrins in the production of large porous particles: development of dry powders for the sustained release of insulin to the lungs." Eur J Pharm Sci **28**(5): 423-432.
Upadhyay, D., S. Scalia, et al. (2012). "Magnetised thermo responsive lipid vehicles for targeted and controlled lung drug delivery." Pharm Res **29**(9): 2456-2467.
van der Velden, V. H. (1998). "Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma." Mediators Inflamm **7**(4): 229-237.
van Vlerken, L. E., T. K. Vyas, et al. (2007). "Poly (ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery." Pharmaceutical research **24**(8): 1405-1414.
Vandevanter, D. R. and D. E. Geller (2011). "Tobramycin administered by the TOBI((R)) Podhaler((R)) for persons with cystic fibrosis: a review." Med Devices (Auckl) **4**: 179-188.
Vemula, P. K., N. Wiradharma, et al. (2013). "Prodrugs as self-assembled hydrogels: a new paradigm for biomaterials." Curr Opin Biotechnol.

Veronese, F. M., O. Schiavon, et al. (2005). "PEG-doxorubicin conjugates: influence of polymer structure on drug release, *in vitro* cytotoxicity, biodistribution, and antitumor activity." Bioconjug Chem **16**(4): 775-784.
Vujanic, A., J. L. Wee, et al. (2010). "Combined mucosal and systemic immunity following pulmonary delivery of ISCOMATRIX adjuvanted recombinant antigens." Vaccine **28**(14): 2593-2597.
Wanakule, P., G. W. Liu, et al. (2012). "Nano-inside-micro: Disease-responsive microgels with encapsulated nanoparticles for intracellular drug delivery to the deep lung." J Control Release **162**(2): 429-437.
Wanakule, P., G. W. Liu, et al. (2012). "Nano-inside-micro: Disease-responsive microgels with encapsulated nanoparticles for intracellular drug delivery to the deep lung." Journal of Controlled Release **162**(2): 429-437.
Wanakule, P., G. W. Liu, et al. (2012). "Nano-inside-micro: disease-responsive microgels with encapsulated nanoparticles for intracellular drug delivery to the deep lung." Journal of Controlled Release.
Wang, J. J., Z. W. Zeng, et al. (2011). "Recent advances of chitosan nanoparticles as drug carriers." Int J Nanomedicine **6**: 765-774.
Watts, A. B., Y. B. Wang, et al. (2013). "Respirable low-density microparticles formed in situ from aerosolized brittle matrices." Pharm Res **30**(3): 813-825.
Weers, J. G., J. Bell, et al. (2010). "Pulmonary formulations: what remains to be done?" Journal of aerosol medicine and pulmonary drug delivery **23**(S2): S-5-S-23.
Westbrock-Wadman, S., D. R. Sherman, et al. (1999). "Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability." Antimicrob Agents Chemother **43**(12): 2975-2983.
Westerman, E. M., A. H. De Boer, et al. (2007). "Dry powder inhalation of colistin in cystic fibrosis patients: a single dose pilot study." J Cyst Fibros **6**(4): 284-292.
Wiegand, I., K. Hilpert, et al. (2008). "Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances." Nat Protoc **3**(2): 163-175.
Winther, B. (2011). "Rhinovirus infections in the upper airway." Proc Am Thorac Soc **8**(1): 79-89.
Woodward, T. C., R. Brown, et al. (2010). "Budget impact model of tobramycin inhalation solution for treatment of *Pseudomonas aeruginosa* in cystic fibrosis patients." J Med Econ **13**(3): 492-499.
Wright, D. H., G. H. Brown, et al. (2000). "Application of fluoroquinolone pharmacodynamics." Journal Of Antimicrobial Chemotherapy **46**(5): 669-683.
Wu, L., O. Zaborina, et al. (2004). "High-molecular-weight polyethylene glycol prevents lethal sepsis due to intestinal *Pseudomonas aeruginosa*." Gastroenterology **126**(2): 488-498.
Wu, Y., C. Liu, et al. (2008). "A new biodegradable polymer: PEGylated chitosan-g-PEI possessing a hydroxyl group at the PEG end." Journal of Polymer Research **15**(3): 181-185.
Xu, X., A. K. Jha, et al. (2012). "Hyaluronic Acid-Based Hydrogels: from a Natural Polysaccharide to Complex Networks." Soft Matter **8**(12): 3280-3294.
Xu, X., X. D. Zhou, et al. (2012). "Tea catechin epigallocatechin gallate inhibits *Streptococcus mutans* biofilm formation by suppressing gtf genes." Arch Oral Biol **57**(6): 678-683.
Yamada, K., N. Kamada, et al. (2005). "Carrageenans can regulate the pulmonary absorption of antiasthmatic drugs and their retention in the rat lung tissues without any membrane damage." Int J Pharm **293**(1-2): 63-72.
Yamamoto, H., Y. Kuno, et al. (2005). "Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions." J Control Release **102**(2): 373-381.
Yang, H. and S. T. Lopina (2003). "Penicillin V-conjugated PEG-PAMAM star polymers." J Biomater Sci Polym Ed **14**(10): 1043-1056.

Yang, W., J. I. Peters, et al. (2008). "Inhaled nanoparticles--a current review." Int J Pharm **356**(1-2): 239-247.
Yang, Y., N. Bajaj, et al. (2009). "Development of highly porous large PLGA microparticles for pulmonary drug delivery." Biomaterials **30**(10): 1947-1953.
Yang, Y., M. D. Tsifansky, et al. (2010). "Inhalable antibiotic delivery using a dry powder co-delivering recombinant deoxyribonuclease and ciprofloxacin for treatment of cystic fibrosis." Pharmaceutical research **27**(1): 151-160.
Yeo, Y., C. B. Highley, et al. (2006). "In situ cross-linkable hyaluronic acid hydrogels prevent post-operative abdominal adhesions in a rabbit model." Biomaterials **27**(27): 4698-4705.
Yildiz, A., E. John, et al. (2012). "Inhaled extended-release microparticles of heparin elicit improved pulmonary pharmacodynamics against antigen-mediated airway hyper-reactivity and inflammation." Journal of Controlled Release **162**(2): 456-463.
Yoncheva, K., I. Doytchinova, et al. (2012). "Preparation and evaluation of isosorbide mononitrate hydrogels for topical fissure treatment." Curr Drug Deliv **9**(5): 452-458.
Zabner, J., S. H. Cheng, et al. (1997). "Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia in vivo." J Clin Invest **100**(6): 1529-1537.
Zaman, M., P. Simerska, et al. (2010). "Synthetic polyacrylate polymers as particulate intranasal vaccine delivery systems for the induction of mucosal immune response." Curr Drug Deliv **7**(2): 118-124.
Zeng, X., G. Martin, et al. (1995). "The controlled delivery of drugs to the lung." International journal of pharmaceutics **124**(2, 3): 149-165.
Zhang, Q., Z. Shen, et al. (2001). "Prolonged hypoglycemic effect of insulin-loaded polybutylcyanoacrylate nanoparticles after pulmonary administration to normal rats." Int J Pharm **218**(1-2): 75-80.
Zhang, W., H. Yang, et al. (2005). "Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene." Nat Med **11**(1): 56-62.
Zhou, J., W. Y. Zhao, et al. (2013). "The anticancer efficacy of paclitaxel liposomes modified with mitochondrial targeting conjugate in resistant lung cancer." Biomaterials **34**(14): 3626-3638.
Zimmermann, U., G. Klöck, et al. (1992). "Production of mitogen-contamination free alginates with variable ratios of mannuronic acid to guluronic acid by free flow electrophoresis." Electrophoresis **13**(1): 269-274.

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